THE RELATIONSHIP OF CYSTEINE-RICH INTESTINAL PROTEIN TO CELLULAR HOST DEFENSE

By

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To Randal, for encouraging me throughout this work and for making me laugh.

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TABLE OF CONTENTS

ABBREVIATIONS

BSA	bovine serum albumin
CAST	cyclic amplification and selection of targets
CONV	conventional
(h, r)CRIP	(human, rat)cysteine-rich intestinal protein
CRIP-Tg	CRIP transgenic mouse
CRP	cysteine-rich protein
DTH	delayed-type hypersensitivity
ELISA	enzyme-linked immumosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GALT	gut-associated lymphoid tissue
H&E	hemotoxylin and eosin
Ig	immunoglobulin
IL-	interleukin-
IFN	interferon
i.p.	intraperitoneal
KO	knockout
LMO	LIM-only
LPS	lipopolysaccharide
MDCK	Madin Darby canine kidney
MLP	muscle LIM protein
MMNC	milk mononuclear cell
MRE	metal response element
MT	metallothionein
NF	nuclear factor
Non-Tg	nontransgenic control mouse
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PHA	phytohemaglutinin
PBMC	peripheral blood mononuclear cell
SPF	specific pathogen free
TBS	tris-buffered saline
TNF	tumor necrosis factor

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Cysteine-rich intestinal protein (CRIP) is a zinc-finger protein which has been identified in several tissues and cells. CRIP mRNA expression is developmentally regulated in the rat intestine, with levels increasing to that of the adult by the time of weaning. The goal of my dissertation project was to utilize two available research models (humans and transgenic mice) in order to learn more about the function of CRIP. A sensitive, enzyme-linked immunosorbent assay (ELISA) was developed which allowed the quantification of CRIP protein in these models.

CRIP mRNA and protein expression was evaluated over the first 6 weeks of lactation in human milk mononuclear cells. There was a trend

toward a decrease in CRIP mRNA expression over the period of lactation examined. This trend was also true for subjects examined longitudinally for CRIP protein expression.

The other model which was utilized in this project was a transgenic mouse model which overexpresses the rat CRIP gene. Two of the main areas which were examined with the transgenic mice were the effect of environment and acute immune challenges (endotoxin and influenza virus) on CRIP expression. CRIP expression was higher when mice were placed in a conventional environment as compared to a specific pathogen free (SPF) environment. The CRIP transgenic mice were more sensitive to both endotoxin and influenza virus. There was also a shift in cytokine production in stimulated mice. CRIP-Tg mice produced more IL-10 and IL-6 and less IL-2 and IFN-γ as compared to Non-Tg mice.

Additionally, the effect of zinc deficiency on CRIP expression in CRIP transgenic and non-transgenic control mice was examined since the zinc fingers could be targets of dietary zinc restriction. Zinc deficiency did not have a marked effect on CRIP levels in the transgenic mice. However, when CRIP expression was examined in metallothionein-null mice, there was an effect of metallothionein levels on CRIP protein. The ability to express increased amounts of CRIP is apparently related to the metallothionein pool in the mouse.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Introduction

Cysteine-rich intestinal protein (CRIP) is a zinc-finger protein which has been identified in several tissues and cells. CRIP mRNA expression is developmentally regulated in the rat, with levels increasing to that of an adult rat by the time of weaning (Birkenmeier and Gordon, 1986; Levenson et al., 1993). CRIP is a member of the LIM-only family of proteins (Dawid et al., 1998) and as such has a LIM domain. This feature consists of two zinc-finger motifs that have a highly conserved CCHC plus CCCC sequence. Being the most elementary of the LIM-only proteins, CRIP has a single LIM motif. Although no clear biological role for CRIP has been defined, experimental evidence suggests that CRIP may function in immune cell activation (Hallquist et al., 1996) and/or in cellular proliferation or differentiation (Dawid et al., 1993).

The goal of this dissertation project was to utilize two available research models to learn more about the function of CRIP. Additionally, a sensitive, immunochemical assay was developed which allowed for quantification of CRIP protein in these models. The development of this

assay is of relevance for two important reasons: (1) no other LIM protein has been quantified in this manner and (2) this assay may serve as an assay for zinc status. Zinc is a nutrient for which status is difficult to evaluate. Since CRIP is a zinc-containing protein, accurate quantification of the protein may help us to better assess zinc status and enhance our understanding of the nutritional significance of zinc.

The first modelutilized in the project was the human and in particular human milk. Currently, several research groups have demonstrated the nutritional and immunological importance of human milk for the human infant. Mononuclear cells in human colostrum and mature milk have the potential to produce several different cytokines (Skansen-Saphir et al., 1993) and thus may be a likely site for CRIP expression and function. By knowing whether CRIP is produced in human milk and the protein's pattern of expression over the first few weeks of lactation, we may better understand CRIP's role in immune function and/or development.

The other model which was utilized in this project was a transgenic mouse model which overexpresses the rat CRIP gene (Davis et al., 1998a). This transgenic model overexpresses the CRIP gene with an expression profile that mirrors that of the endogenous gene. Thus the greatest amount of expression occurs in tissues which already produce the largest amount of CRIP mRNA and protein (such as small intestine, spleen, thymus, and lung).

Because CRIP is expressed so highly in immune cells and tissues, the immunological relevance of this protein is of great interest. Two of the main areas examined with the transgenic mice were the effect of environment and more direct immune challenges (endotoxin and influenza virus) on CRIP expression.

The murine overexpression model is also important because although CRIP is a zinc-containing protein (binds two zinc atoms per LIM motif), its expression cannot be induced by dietary zinc (Levenson et al., 1994b). However, whether dietary zinc intake has an effect on overexpression (threefold to sevenfold in certain tissues) is a question which was addressed in this project. Dietary zinc status has been shown to play a central role in immune function with zinc-deficient persons demonstrating increased susceptibility to several pathogens (Shankar and Prasad, 1998). Consequently CRIP expression could be a factor in zinc-related immune dysfunction.

The nutritional relevance of this project is strongly related to immune function. Some controversy exists over whether certain micronutrient deficiences have direct or indirect effects on the function of immune cells (Thurnham, 1997). However, there is a great deal of evidence which supports the use of zinc supplementation for the treatment of diarrhea (Fuchs, 1998) and improvement of gut integrity (Roy et al., 1992). Several

pieces of evidence have pointed toward a role of CRIP in immune function and that area of investigation was the focus of this project.

Objectives

1. To develop a sensitive immunochemical assay (enzyme-linked immunoabsorbent assay or ELISA) for the detection and quantification of CRIP protein.
2. To develop information needed to define a function for CRIP:
   a. by studying the expression of CRIP in human milk mononuclear cells by examining CRIP mRNA and proteins levels and evaluating the pattern of expression during lactation.
   b. by examining CRIP expression in various tissues and cells of CRIP transgenic mice during different stages host defense and during altered zinc status.

Hypothesis

The expression of CRIP in immune cells and tissues is indicative of a role for this protein in cellular host defense.

Literature Review

Cysteine-Rich Intestinal Protein Cysteine-rich intestinal protein (CRIP) cDNA was identified in 1986 by Birkenmeier and Gordon. This protein is 77 amino acids in length and has as a high cysteine content (11%). Using high

resolution gel filtration chromatography and a Western blot-^{65}Zn assay, Hempe and Cousins (1991) demonstrated that CRIP binds exogenous zinc. This metal-binding property of CRIP has been attributed to a LIM motif which is the cysteine-rich sequence $CX_2CX_{17}HX_2CX_2CX_2CX_{17}CX_2C$ (where C = cysteine, X = amino acid, H = histidine). This motif folds to form zinc fingers (Li et al., 1991). Unlike the original LIM proteins which were characterized (Lin-11, Isl-1, and Mec-3, Freyd et al., 1990), CRIP does not contain a homeodomain, or conserved DNA-binding region. It is considered a LIM-only protein because it lacks this feature. LIM-only proteins have been suggested to play a role in cell proliferation and differentiation (Dawid et al., 1995). Thus far, highest levels of CRIP mRNA have been detected in the small intestine (Birkenmeier and Gordon 1986, Levenson et al. 1993, Khoo and Cousins 1994). However, recent data shows that CRIP levels in the small intestine and peritoneal macrophage are comparable (Hallquist et al., 1996) which suggests that CRIP may play a role in immune cells. Since the intestine is also the largest immune organ in the body, an immune function for CRIP in the intestine is also possible.

In the rodent, CRIP appears to be under developmentally-influenced regulation. In their original work, Birkenmeier and Gordon examined CRIP mRNA levels in the rat small intestine during fetal and postnatal periods. They detected CRIP mRNA at day 21 of fetal development, with an

additional increase during the first two days postpartum. They then noticed a marked increase in CRIP to peak level which corresponded with the time of weaning. Additionally, they detected a decrease in CRIP mRNA of 40% at day 35. Subsequent studies by our research group has confirmed this same pattern (Levenson et al., 1993), but they have not detected the same drop in CRIP mRNA at day 35. These same patterns of CRIP expression have not been examined in the mouse or human.

The zinc-binding properties of CRIP and its high levels in the small intestine initially led researchers to speculate that it was involved in transmucosal zinc transport (Hempe and Cousins, 1991, 1992). However CRIP expression was not found to be regulated by zinc. A study in which rats were fed a zinc-supplemented or zinc-deficient diet revealed that zinc did not regulate CRIP mRNA levels in the intestine (Levenson et al., 1994b). In addition, zinc also did not activate a CRIP promoter/CAT reporter gene construct in transfected IEC-6 cells (Levenson et al., 1994b).

Further studies to identify potential functional roles for CRIP have looked at the effect of hormones on CRIP expression. Needleman et al. (1993) examined administration of the glucocorticoid, dexamethasone on the expression of CRIP in neonatal rat jejunum. Dexamethasone treatment resulted in increased expression of CRIP mRNA and protein. Similar response to dexamethasone treatment in neonatal rat intestine was noted by

Levenson et al. (1993). Because of this response to glucocorticoid in vivo, the CRIP promoter was used to examine a reporter construct in transfected IEC-6 cells (Levenson et al., 1994b). Several sequences that exhibit a high amount of homology to glucocorticoid response elements were discovered.

As mentioned previously, CRIP may also serve a role in immune cells. This observation argues against a function for CRIP only in the intestine. Hallquist et al. (1996) used lipopolysaccharide (LPS) to induce an acute-phase immune response in rats. Administration of LPS resulted in an increase in CRIP mRNA levels in the spleen, intestine, peritoneal macrophages, and PBMC. This study suggests that CRIP may aid in immune cell activation or differentiation.

Although no clear biological role exists for CRIP, much evidence suggests a role in cell proliferation and differentiation. Additional investigation is needed to define CRIP's biological role. A CRIP transgenic mouse line was developed in our lab (Davis et al., 1998a). This transgenic line has prove to be a useful tool in learning more about CRIP (see Chapter 4).

LIM Proteins The LIM family of proteins began with the grouping of three transcription factors which were involved in developmental regulation. These three LIM proteins which were characterized inlcluded *lin-11, isl-1, and mec-3* (Freyd et al., 1990; Karlssson et al., 1990). For example, *mec-3*

is a *Caenrhabditus elegans* gene required for differentiation of mechanosensory neurons (Way and Chalfie, 1988). Interestingly, after this class of proteins was created, it was noted that the first LIM domain sequence which had been reported was contained in the cysteine-rich intestinal protein, CRIP (Birkenmeier and Gordon, 1986). Since this time more than 60 gene products have been identified that contain from one to five copies of the LIM domain (Sanchez-Garcia and Rabbitts, 1994).

There are considered to be two or three classes of LIM domains, based upon sequence comparisons (Jurata and Gill, 1998; Dawid et al., 1998). Both groups agree that there are LIM homeodomain proteins and LIM-only proteins. LIM homeodomain proteins (LHX) contain a conserved DNA-binding region (homeodomain). The LIM-only proteins do not have a homeodomain. CRIP is a member of the LIM-only group. Dawid et al. (1998) groups the LIM domains based largely on structural features rather than whether there is a homeodomain present. In the first group, N-terminal LIM domains always occur in tandem. In the second group, the proteins are composed largely of LIM domains (includes CRIP). In the third group, the proteins contain different numbers of LIM domains located in the C-terminus.

As a whole, the classification of LIM proteins is currently undergoing nomenclature standardization. For example, the nuclear LIM-only proteins

are now being designated LMO which replaces the original names of Rhombotins (Rbtn; Greenberg et al., 1990) and T cell translocation genes (Ttg; McGuire et al., 1989). At present, no convention has been adopted for the extra-nuclear LIM-only proteins such as CRIP.

Much interest has been placed on the function of LIM proteins. The exact function of any of the LMO proteins has yet to be established. LIM proteins may increase in number with complexity of the organism since, in *C. elegans* and *Saccharomyces cerevisiae*, the LIM domain represents 6% and 2% of the zinc finger domain genes in the respective genomes (Clarke and Berg, 1998, and Jurata and Gill, 1998). Consequently, a regulatory function is likely.

The most commonly proposed function of the LIM proteins is being multiple binding and adapter modules. The proteins which contain only short regions beyond the LIM domain may function solely as adapters to bring other components together in a complex. CRIP may fall into this category. Other LIM proteins contain additional motifs which can mediate interactions with various proteins. For example, the LIM domain of zyxin can bind another LIM domain on cysteine-rich protein (CRP) and α-actinin (Sadler et al., 1992, Schmeichel and Beckerle,1994). It has been proposed that the ability of zyxin to assemble proteins could mediate integrin-dependent signaling events that lead to cell differentiation or modulation of

cytoarchitecture (Macalma et al., 1996). The LIM-only proteins as a group may prove to be even more crucial for developmental processes as the importance of coregulatory proteins in the process of gene transcription are being explored (Mannervik et al., 1999). Because CRIP is a LIM-only protein, the focus of this project will be on proposed functions of this group of LIM proteins.

Functions of LIM-Only Proteins The LIM-only proteins can be further divided into 2 groups; nuclear and extranuclear. While there has been a large body of research focused on the nuclear LIM-only proteins, less attention has been focused on the extranuclear LIM-only proteins. As pointed out above, the nuclear LIM-only proteins have been designated as the LMO group, consisting of 4 different proteins (LMO 1-4). It is thought that these proteins play a role in transcription as they associate with other known transcription factors in the nucleus. The mechanism(s) by which they participate in transcription are not clear.

The extranuclear proteins include CRIP and CRP. As mentioned above, CRP binds with zyxin through LIM-LIM interactions and this binding may be necessary for cell differentiaion. CRP may also be required for muscle differentiation (Crawford et al., 1994). The function of CRIP is the focus of this project.

The first member of the LMO group, LMO1 was identified as a gene disrupted by a T-cell translocation event in cells derived from a patient with T-cell acute lymphoblastic leukemia (T-ALL; Boehm et al., 1988). This protein is highly expressed in the embryonic and adult nervous systems of the mouse (Greenberg et al., 1990). Lower levels of expression are seen in the developing thymus. The second member of the LMO family, LMO2, has been widely researched and will be discussed separately below. The third member, LMO3, was identified by close homology to LMO1 (89% identity) (Foroni et al., 1992). Like LMO1, LMO3 is highly expressed in nervous system tissues, but LMO3 does not appear to be related to leukemia translocation events. The final member, LMO4 has been identified as a gene with a novel pattern of expression during embryogenesis (Kenny et al., 1998). This LMO protein is distinguished from the others in that it is also expressed in mature T-cells.

One of the most widely examined LMO proteins is LMO2. This protein is 50% identical to LMO1, with the highest amount of homology in the LIM domains and linker region (Boehm et al., 1991; Royer-Pokora et al., 1991). Unlike the other LMO proteins, LMO2 expression is widespread. The mRNA is highest in embryonic liver and spleen, the sites of fetal hematopoiesis, as well as in adult brain, kidney, spleen, and liver. Like LMO1, LMO2 is activated following specific translocations in T-cells, leading

to the pathogenesis of T-ALL. However, these particular types of translocations which activate LMO2 are found more frequently in T-ALL patients. This suggests that LMO2 protein may play a more important role in the development of this form of leukemia. The mechanism(s) by which LMO2 functions in this process have been explored (Larson et al, 1996).

As pointed out earlier, it has been suggested that LIM-only proteins act as adapters which bring together other components into a complex. Some of the components which LMO2 bring together have been identified. When LMO2 dimerizes with Tal1 (a protein important for erythroid development), thymocyte development is altered and T-cell tumorigenesis is promoted (Larson et al., 1996). Transgenic mice which co-express LMO2 and Tal1 genes in T-cells developed T-cell tumors approximately 3 months earlier than mice which only expressed LMO2 transgene. The synergistic action of the two proteins was necessary in order for increased tumorigenesis to occur.

Techniques such as CASTing (cyclic amplification and selection of targets), histochemistry, and band shift assays have been used to elucidate the other protein partners of LMO2 (Wadman et al., 1997; Grutz et al., 1998; Jurata et al, 1996). At present, LMO2 is considered to be a bridging molecule which assembles Tal1, E47, GATA-1 and Ldb1/NLI proteins. The

complex formed from these proteins binds to DNA and plays an important role in erythropoesis (Warren et al., 1994; Wadman et al., 1997).

Warren et al. (1994) demonstrated the importance of LMO2 in erythroid development by homozygous deletion of the LMO2 gene in mice. This deletion resulted in lethality at approximately E10.5. Examination of the developing embryos prior to this point revealed that there were no erythroid cells in the yolk sac or the fetal circulation. By analysis of mouse embryonic stem cells, this group later demonstrated that LMO2 is necessary for early stages of hematopoiesis, perhaps even before the development of the bone marrow stem cell (Yamada et al., 1998).

Taken together, data suggest that the LMO and other LIM-only proteins appear to share certain functional characteristics. They join together with other proteins (including other LIM proteins) and transcription factors, acting most commonly as bridging molecules. In the case of the LMO proteins, they appear to play a role in transcription through interaction with transcription factors. The exact mechanism of this interaction and target genes must still be described. The most common characteristic that the LIM-only proteins share is that they appear to function in development and differentiation. Whether CRIP can be included in this category is yet to be defined and is the focus of this project.

Zinc in Nutrition There is some debate over how much zinc is consumed by the U.S. population. Some sources place the consumption level at 47%-67% of the RDA (Solomons and Cousins, 1984) whereas others estimate consumption to be higher at around 81%-90% (Moser-Veillon, 1990). The most recent edition of the Recommended Dietary Allowances takes into consideration zinc needs based upon sex, age, pregnancy, and lactation (National Research Council, 1989). The current RDA for zinc is 15 mg/day for males over 10 years, 12 mg/day for females over 10 years, 15 mg/day during pregnancy, 19 and 16 mg/day for lactation during the first and second 6 months, respectively, 5 mg/day for infants, and 10 mg/day for children under 10 years. These recommendations assume a fractional absorption of 20%.

Zinc in foods is most abundant in animal products such as red meat, milk, and seafood (Smolin and Grosvenor, 1994). There is also some zinc contained in grains, but this zinc is more likely to be bound by phytates which decrease zinc absorption. Some of the earliest studies concerning zinc deficiency in humans were on populations which had a diet consisting primarily of grain products with very little animal products consumed. In the U.S. population, it is estimated that 70% of the zinc consumed comes from animal products (Welsh and Marston, 1982). Other factors in food which may decrease zinc absorption include oxalate, fiber, calcium

supplementation, EDTA, and tannins (Sandstrom and Lonnerdal, 1989; Wood and Zheng, 1995). There are also components of food which increase the bioavailability of zinc. For example, the zinc in human milk is more bioavailable than in cow's milk. This increased bioavailability is most likely related to differences in protein composition.

Zinc is tightly regulated in human milk. Several studies in which the effect of zinc supplementation in lactating women has been examined have failed to find any correlation between maternal dietary zinc intake and milk zinc concentration (Lonnerdal, 1986; Moser-Veillon and Reynolds, 1990; Krebs et al., 1995). However, there remains a question as to whether zinc supplementation during lactation in populations with chronically low dietary zinc intakes may have some benefit (Krebs, 1998).

On average, there is 2-3 mg Zn/day present in milk during the early weeks postpartum. Zinc quantity in milk drops to around 1 mg Zn/day by 2-3 months postpartum. There is some question as to whether the zinc supplied by breastmilk alone is adequate for the infant (Allen, 1998) and there is evidence that zinc supplementation in breast-fed infants can increase growth velocity (Krebs et al., 1996; Walravens et al., 1992). In addition to the fact that maternal zinc supplementation does not appear to strongly influence milk zinc content, maternal infection does not appear to

affect milk zinc levels (Zavaleta et al., 1995). Currently, little is known about the effect of zinc deficiency and milk zinc levels.

Functions of Zinc Zinc is an element which plays many roles in the body. It has been characterized as having three different functions-regulatory, catalytic, and structural (Cousins, 1996). When referring to the regulatory aspects of zinc, the most common role is that of gene expression regulation (Cousins, 1999). The regulation of the metallothionein gene by zinc is a clear example of this process (Cousins, 1994). Zinc affects metallothionein transcription by binding to a transcription factor which in turn recognizes specific sequences (metal response elements; MRE) on the methallothionein promoter. This process has been observed in many models including rats (Cousins and Lee-Ambrose, 1992) and humans (Grider et al., 1990; Sullivan and Cousins, 1998). For example, when human subjects received 50 mg/day of zinc supplement, monocyte metallothionein mRNA levels were elevated after 1 day of supplementation and returned to basal levels within 4 days of supplementation (Sullivan and Cousins, 1998). One of the primary goals of this area of research (i.e. zinc's effect on gene regulation) is the development of a functional indicator of zinc status. Alternatively, there is interest in the more global role zinc may play in regulating the gene expression machinery and thus developmental biology (Falchuk, 1998). Another interesting aspect of zinc and gene regulation is, as a component of

zinc finger proteins such as LMOs, its potential to play some part in the organization of coregulatory proteins which affect transcription. This aspect will be explored further below.

Zinc also has a catalytic role, being essential for enzymes from all six classes of enzymes (Valee and Galdes, 1984). Taking into consideration that the same metalloenzymes have been characterized for different organisms, there are still over 50 zinc-containing enzymes. These enzymes have decreased activity when zinc is removed, and repletion with zinc restores activity. Examples include carbonic anhydrases, alkaline phosphatases, RNA polymerases I, II, and III, and alcohol dehydrogenase. The later example is a good example of how activity of a zinc metalloenzyme can affect the development of a disease, in this case alcoholic liver disease (Hambidge et al., 1986).

The final general function of zinc is as a structural component of enzymes and other proteins. Not only does zinc play a catalytic role in some enzymes (as mentioned above), but this metal also influences the structure for some enzymes. An example is the cytosolic enzyme CuZn superoxide dismutase (Cu/Zn SOD) where zinc has a structural role and copper plays a catalytic role. Another important structural role for zinc is in the zinc finger motif of proteins. In this motif, zinc forms a tetrahedral complex with four cysteines and sometimes histidine. The general

structure is -C-X_2-C-X_n-C-X_2-C-, where C represents cysteine and X represents other amino acids. It is estimated that up to 300-700 human genes code for these proteins. Considering that this represents 1% of the human genome and that dietary zinc may affect the function of some of these proteins make this an interesting area of nutrition science to focus on. As pointed out above, CRIP is a zinc finger protein and the effect of dietary zinc (in particular zinc deficiency) and zinc from another metalloprotein (metallothionein) will be explored as part of this dissertation project.

Zinc Deficiency It has been known since the late 19th century and the early 20th century that zinc is nutritionally essential for higher forms of plant life and several different animals. In the early 1960s, work by Prasad and colleagues established that zinc deficiency can occur in humans with symptoms of marked hypogonadism, short stature, rough and dry skin, mental lethargy and hepatosplenomegaly (Prasad et al., 1979) More recent work in the area of zinc points to a decrease in immune function with zinc deficiency (Keen and Gershwin, 1990). Although the exact biochemical basis for zinc's role in immune function has not been established, the mechanism may relate to the basic functions of zinc listed above. There is a great deal of research focusing on zinc deficiency and immune function in mice. Since this model will be used, this literature will be reviewed below.

Zinc Deficiency and Immune Function There are several groups who have reported the effects of zinc deficiency and immune function (Prasad, 1998, Shankar and Prasad, 1998, Kubena and McMurray, 1996, Wellinghausen and Rink, 1998, and Wellinghausen et al., 1997). Each of these groups agrees that zinc status affects thymic function, lymphoproliferation, T-lymphocyte development, and resistance to infections. Zinc can affect several mediators of host immunity, ranging from the skin's function as a barrier to cellular and humoral immunity. One way to understand zinc's role in immune function is to consider two processes in which zinc has been shown to exert some effect: 1) development of immune cells and 2) activation and proliferation of immune cells.

Studies of zinc deficiency in mice have described reductions in thymus size (Fraker et al., 1977; Fraker et al., 1978). The thymus is the central organ for T lymphocyte development. Additionally, T lymphocytes were progressively depleted in spleen, lymph nodes, and peripheral blood in zinc-deficient mice (Fraker et al., 1986). There are also changes in B lymphocyte populations in zinc-deficient mice (King et al., 1995). This group observed declines in cells of the B lineage in bone marrow of 43% and 91% in moderately zinc-deficient and severely zinc-deficient mice, respectively.

Zinc deficiency also affects lymphocyte development in the thymus and bone marrow. The function of these immune cells is also affected. The ability of zinc-deficient mice to generate a T-helper response to immunization with sheep erythrocytes was decreased as compared to zinc-adequate mice (Fraker et al., 1978). However, T-helper function was regenerated when these zinc-deficient mice were returned to a zinc-adequate diet. James et al. (1987) found that mice which were placed on a zinc-deficient diet produced splenic macrophage which were not able to help mediate mitogen induced proliferation of splenic T-cells. This group also found that the proliferative capacity of the purified T-cells was not different from controls when the T-cells were combined with macrophage from pair-fed animals. Zinc deficiency caused decreased T-cell function in zinc-deficient mice as observed by a decrease in IL-4 and IL-5 production (Shi et al., 1998). In regards to a response to specific pathogens, zinc is required for B-lymphocyte and cytokine response to lipopolysaccharide (Driessen et al., 1995). Finally, zinc deficiency causes decreased natural killer cell activity in both humans and other animals (Shankar and Prasad, 1998).

The studies described here represent a small portion of the many studies which have looked at zinc deficiency and immune function. Because there is evidence that CRIP plays some role in immune function, we chose

to evaluate the effect of zinc deficiency on CRIP expression in CRIP-Tg and Non-Tg mice (Chapter 7).

Zinc, Immunity, and the Elderly The elderly population in America is growing at a rapid rate. It is estimated that by the year 2030, the population of persons 65 or older will more than double (Ausman and Russell, 1999). With such a large elderly population, the focus of nutrition-related research may shift to expand our knowledge base of this group. A primary goal would be improving the quality of life through nutrition. One aspect of elderly health that is affected by nutrition is immune function (Buzina-Swooticanec, 1998). It is generally accepted that immune function declines with age, making the elderly more susceptible to infections such as influenza.

One nutrient in particular which is linked with immune function in both young and aged populations is zinc (Prasad, 1998, Shankar and Prasad, 1998). The question arises whether poor zinc status affects immune function in the elderly. When insitutionalized elderly men and women (n = 756) were monitored for micronutrient status, there was a high prevalence of low serum zinc (Monget et al., 1996). Some research groups have pointed to the fact that marginal zinc deficiency is hard to measure in the elderly, especially since definitive indicators of zinc status are lacking

(Bales et al., 1994). Clearly the need for a definitive status indicator is relevant to this population as well as others.

When monitoring average zinc consumption in the diet, the elderly consume 7-10 mg (women and men, respectively) on a daily basis which falls below the 1989 RDA of 15 mg/day for men and 12 mg/day for women (Pilch and Senti, 1984). Like other minerals such as calcium, zinc absorption is thought to generally decline with age, although some research groups have demonstrated little if any effect of age on absorption (Couzy et al., 1993). Whether absorption is affected by age or not would be a secondary problem considering that actual intake is low.

Some research has focused on the effect of nutrition on cell-mediated immunity in the elderly. In general, aging is associated with a decrease in the ratio of mature to immature T and B lymphocytes, and decline in the T-helper 1 subset (Th1) along with an increase in the Th2 subset. There is also an increase in the proportion of naive/memory lymphocytes. This results in weaker cell-mediated immune function. There is some evidence which demonstrates that supplementation with high pharmacologic doses of certain nutrients (such as zinc and vitamin E) may help to improve immune responses in the elderly (Lesourd, 1997). If the zinc-finger protein CRIP plays a role in some aspect of host immunity, CRIP levels in the elderly may be a consideration for a zinc status indicator.

Host Immunity. The process of dealing with different pathogens which an organism encounters in its environment (host immunity) can be made more understandable by considering that there are two general mechanisms by which the pathogens are processed. Pathogens or their products are divided into two categories: 1) intracellular pathogens or 2) extracellular pathogens (Janeway and Travers, 1994).

Resistance to intracellular pathogens such as viruses is dominated by cell-mediated forms of immunity. Specifically, T-cells recognize and attack cells infected with intracellular pathogens. Extracellular pathogens and their toxins are eliminated by antibodies (humoral immunity). Thus this type of host immunity is dominated by B lymphocytes which have the potential to make antibodies. Common to both of these types of immunity are $CD4^+$ T helper lymphocytes which produce cytokines that are associated with inflammatory responses or B lymphocyte help (Stout and Bottomly, 1989). These types of T-cells are referred to as: 1) Th1 which produce IL-2, IFN-γ, and TNF-ß that mediate delayed-type hypersensitivity (DTH) responses and 2) Th2 helper cells which produce IL-4, IL-5, IL-6, IL-10, and IL-13 that induce B cells to generate antibodies. A third type of T helper cell, Th0, has been identified as producing cytokines typical of both Th1 and Th2 cells (O'Garra and Murphy, 1996). It is thought that these T helper cells are

important for clearing pathogens where the correct balance of cell-mediated and humoral immunity is needed with a minimum of immunopathology.

In this project, we chose to evaluate cytokine production in CRIP-Tg mice to determine whether CRIP overexpression affected Th1/Th2 cytokine balance. We challenged the mice with two types of pathogens or toxins; lipopolysaccharide (LPS) or phytohemaglutinin (PHA) which are both extracellular and influenza virus which is an intracellular pathogen (Chapters 5 and 6). The mice were evaluated for their ability to confront these pathogens and cytokines were measured.

Cytokine patterns for the Th1 and Th2 cells are distinct, but the effects of these cytokines is not always clearly "Th1" or "Th2" as was previously thought (Muraille and Leo, 1998). Although there is much coregulation between the two types of cells, there is evidence that immune responses mediated by the cytokines are not necessarily polar. This may also be a possible area where Th0 cells are important.

As mentioned previously, Th1 cells are associated with IL-2 and IFN-γ. The differentiation of these T helper lymphocytes is driven by IL-12 and IFN-γ. The differentiation of Th2 cells, which produce IL-4, IL-5, IL-6, and IL-10, is influenced mostly by IL-4. IL-10 may play some role in promoting Th2 cell development, but this effect is secondary to this cytokine's ability to suppress Th1 cells. Additionally, IFN-γ and IL-4 are considered to be

mutually antagonistic in their functions (Paludan, 1998). The balance between Th1/Th2 responses is not always this specific. However, when this balance is not properly achieved in the infected organism and the wrong response is initiated, there is no doubt about the consequences. Balance in host immunity is crucial in minimizing the spread of infectious agents which can cause severe host pathology. For example, insult with lipopolysaccharide will generate a dominantly Th2 response (Rietschel and Brade, 1992). If this response is too severe, septic shock can result.

Although we know a great deal about zinc's roles in immune function, the picture is not complete. It has been proposed that the role that zinc plays in T helper lymphocyte function and cytokine production needs to be explored further. As will be shown in later chapters, it may be possible that CRIP plays some role in regulating Th1/Th2 responses (NIH Report, 1999).

Transcription Factors A transcription factor may be defined as a regulatory molecule with a direct effect on transcription, whose substrate is another macromolecule and not a small molecule (Ouzounis and Papavassiliou, 1997). This effect may be activation or repression of gene expression. Additionally, the regulation can be mediated either by direct binding to DNA or by indirect interactions with other transcription factors. For example, the LIM-only protein LMO2 described earlier is considered to be a bridging molecule which assembles Tal1, E47, GATA-1 and Ldb1/NLI proteins. The

complex formed from these proteins binds to DNA and this binding or lack of influences transcription. Transcription factors are a very heterogenous category of proteins, but are some of the most well-studied proteins.

Nutritional regulation of transcription factors and thus gene regulation is an interesting and growing area in the field of nutrition. Some of the most commonly studied nutrient/gene regulation systems include vitamin D-regulated genes (calcitriol receptor) (Holick, 1994), vitamin A-regulated genes (retinoic acid receptor) (Pfahl and Chytil, 1996), and zinc-regulated genes (metal response elements) (Cousins, 1994). The abundance of transcription factors may also be indirectly influenced by diet (Cousins, 1999). One of the main objectives of this project is to develop information needed to define a function for CRIP. A potential function of CRIP may be as a transcription factor.

It is likely that if CRIP plays some role in transcription, it would be through protein-protein interactions as described above. CRIP does not contain a homeodomain or DNA-binding domain and thus most likely does not have a direct effect on transcription. Additionally, CRIP appears to be primarily a cytosolic protein and work is being done in our lab to confirm the localization. CRIP's role in transcriptional regulation would be similar to that of LMO2, acting as a bridging molecule between other proteins, including proteins that would bind to DNA.

Because of its potential to be involved in protein-protein interactions, CRIP may also be a coregulatory protein that can be recruited to a DNA template by transcription factors. Recent studies in *Drosophila* show that there are coactivators and corepressors that mediate communication between regulatory proteins and the RNA polymerase II (Pol II) complex during transcription (Mannervik et al., 1999). Examples include CREB binding protein, histone acetyltransferase, COOH-terminal binding protein, and the reduced phosphate dependency histone deacetlyase (all examined in *Drosophila*). During development, proteins such as this are critical in determining cell fate.

Transcriptional regulation of cytokine synthesis and function has been extensively studied. The transcription factor, signal transducer and activator of transcription 4 (Stat4), is crucial for IL-12 signalling in natural killer cells. This process is dependent on several enzymes such as type 2 NO synthase for signalling to occur (Diefenbach et al., 1999). Analysis of the promoter for TNF-α has revealed that at least two separate regulatory sites are required for LPS-stimulation of this cytokine. TNF-α promoter contains an overlapping Sp1/Egr-1 site and a region containing CRE and NF-κB sites (Yao et al., 1997). In this example, the LPS-driven production requires the transcription factors Egr-1, c-Jun, and NF-κB. The transcription factor Egr-1 also regulates IL-2 transcription and works synergistically with

the nuclear factor of activated T-cells (NFAT) in this process (Decker et al., 1998).

Transcription factors and coregulatory molecules play important roles in nutrition, development, and immune function. Although a clear function for CRIP has not been determined, suggested roles for the protein also center around these three key areas. Analysis of the CRIP promoter has revealed that there are binding sites for the transcription factors Sp-1 (2 sites), AP-2 (2 sites), and GATA-2 (3 sites) (Levenson et al., 1994a). Functional aspects of these sites and other potential sites of regulation have not been explored further.

Metallothionein and CRIP Intestinal zinc absorption can be divided into two processes: transcellular and paracellular. The transcellular process has several phases for the transport of the metal across enterocytes. Zinc must be taken up at the brush border, move across the cell, pass through the basolateral membrane and finally enter into the plasma. The zinc transporter, ZnT-1, is localized at the basolateral surface and may aid in zinc efflux from enterocytes (McMahon and Cousins, 1998).

Although this process has been well defined, the exact mechanisms of zinc absorption have not been determined. One protein of considerable debate in this process is metallothionein (MT). Metallothioneins are cytosolic, heavy metal-binding proteins with a high cysteine content

(approximately 33%) that can bind 7 atoms of zinc per protein molecule (Dunn et al., 1987). There are four isoforms, with MT-1 and MT-2 being most highly expressed in intestine, liver, pancreas, and kidney. It has been suggested that MT acts as Zn pool to essentially buffer body zinc levels.

In addition to metallothionein, CRIP was originally thought to have a role in intracellular zinc absorption (Hempe and Cousins, 1991). It was later demonstrated that expression of CRIP in the rat intestine was not zinc-dependent (Levenson et al., 1994b). In contrast, MT gene expression in the intestine is dependent upon zinc (Cousins and Lee-Ambrose, 1992). The role for MT in regulating intestinal zinc absorption was further strengthened by examing zinc absorption in metallothionein transgenic and knockout mice (Davis et al., 1998b). In that study, there was an inverse relationship between intestinal metallothionein and serum zinc concentration after an oral zinc dose. There was also a direct relationship between serum zinc and the expression of the zinc transporter ZnT-1.

In addition to its role in zinc absorption, the possibility that MT serves as a pool of zinc for some zinc finger proteins has been explored. Recent in vitro studies have provided further evidence that metallothionein is involved in an equilibrium which favors the donation of zinc ions to apoproteins (Maret et al., 1998). This mechanism has been viewed as a process for coordinating zinc occupancy of apoenzymes (Udom and Brady, 1980).

Furthermore, zinc finger motifs of proteins can be included among the targets of potential zinc donator/acceptor molecules such as MT (Zeng et al., 1991). Zinc bound to MT has been shown to be kinetically labile (Maret et al., 1998). Therefore, depending upon relative Zn(II) binding affinity of the target molecule, MT could act as either a zinc acceptor or zinc donor. Of particular interest are in vitro studies showing zinc from MT is able to activate DNA binding of Tramtrack, a zinc finger transcription factor from *Drosophila* (Roesijadi et al., 1998). Based upon these results, it has been suggested that MT may regulate gene expression through reciprocal zinc exchange with certain transcription factors. These experiments have utilized *in vitro* research models and this process has not been studied in the intact animal.

MT-null (knock out, KO) mice provide a model to examine the function of MT within an integrative context. We used this genotype to examine the consequence MT gene deletion on CRIP levels induced by lipopolysaccharide (LPS). CRIP expression increases with LPS challenge in rats and mice (Hallquist et al., 1996). Using this response in CRIP expression, we hypothesized that if MT serves as a source of zinc for the zinc fingers of CRIP, levels of the protein, as measured by ELISA, should not increase after LPS challenge in the metallothionein knockout mice.

Binding of ^{65}Zn in CRIP is presumably via the protein's two zinc fingers. Kosa et al. (1995) have provided convincing data that the two zinc fingers have dissimilar zinc binding affinities, with the CCHC finger having the more facile zinc affinity. It is likely that this is the target for ^{65}Zn binding observed in *in vivo* experiments (Hempe and Cousins, 1991). These observations also open the possibility that one or both of the fingers of the LIM domain exchange their zinc with intracellular ligands in a coordinated or non-coordinated fashion to influence folding of the protein. However, the exact origin of the zinc donated to apo zinc finger proteins is not known, but as mentioned has been the focus of recent research.

CHAPTER 2
DEVELOPMENT OF CRIP ELISA

Introduction

The first objective of this dissertation project was to develop a sensitive immunochemical assay for the detection and quantification of CRIP. I chose to develop a sandwich enzyme-linked immunosorbent assay (ELISA). The ELISA technique utilizes the interaction between an antigen and an antibody against that particular antigen. In this case, the antigen is CRIP and this protein was used to generate antibodies which could be utilized for an ELISA or other immunochemical techniques.

Although Western analysis is a common immunochemical technique which has been utilized to detect CRIP in various tissues and cells (Fernandes et al., 1997), the ELISA technique has certain advantages. The sandwich ELISA utilizes two antibodies which recognize different epitopes of the CRIP molecule. In contrast, the Western technique utilizes a single antibody capable of recognizing only one epitope. Recognition of more than one epitope in the ELISA increases the specificity of the detection/quantification process and decreases the background. In this ELISA, I have utilized antibodies raised against CRIP in animals which are

phylogenetically distinct (chicken and rabbit). This further ensures that two separate epitopes of the CRIP molecule are being recognized.

Another advantage of the ELISA is that several samples can be quantified on a 96-well microtiter plate. This aspect becomes very important when larger animal or human studies are being conducted and several tissue samples are collected which need to be analyzed at the same time. The samples can also be run in duplicate or triplicate or alternatively, serial dilutions of the unknown samples can be used as a control. These types of controls are difficult or time-consuming to run when the gel- or comb-size of the Western technique is considered.

Finally, the microtiter plates used in the ELISA help to ensure the sensitivity and efficiency of the assay. The microtiter plates used (Corning-Costar, Corning, NY) are coated with a hydrazide surface that ensures proper orientation of the coating or capture antibody. In order to ensure this orientation, the antibody must be activated with sodium periodate. Antibodies have vicinal hydroxyl groups in their Fc region. Upon oxidation with sodium periodate, the hydroxyl groups are converted to dialdehydes. The hydrazide-coated plates have amine groups which can then react with the dialdehydes on the antibody to form a Schiff's base. The antibody is thus oriented in such a manner that the Fab region of the antibody is exposed, allowing for the interaction of the antigen and antibody. This

method is more efficient because the antibodies are aligned uniformly on the plate and more of the antibody can bind to the plate (Figure 2-1). Use of these microtiter plates proved to be a great advantage for this assay.

The final phase in the development of the CRIP ELISA involved sample preparation. During the initial experiments with the ELISA, cytosolic preparations from human milk mononuclear cells or mouse and rat small intestinal mucosa were tested in the CRIP ELISA. These types of samples were in effect used to troubleshoot and standardize the CRIP ELISA method.

When cytosolic samples of mouse spleen and thymus or RAW cells (murine macrophage cell line) were tested, these samples did not work in the assay. Upon performing serial dilutions of these preparations, no corresponding change in absorbance (405 nm) was observed. The absorbance readings for these types of samples were at or only slightly above the background absorbance values. This observation held true whether large or small amounts of cytosolic samples were used in the assay. It appeared that something was interfering with the recognition of the CRIP antigen by the antibodies in the ELISA. This observation raised the question of whether CRIP may have some type of protein partner in some tissues. There is evidence that other LIM-only proteins have protein partners. For example, the protein zyxin is known to have two protein

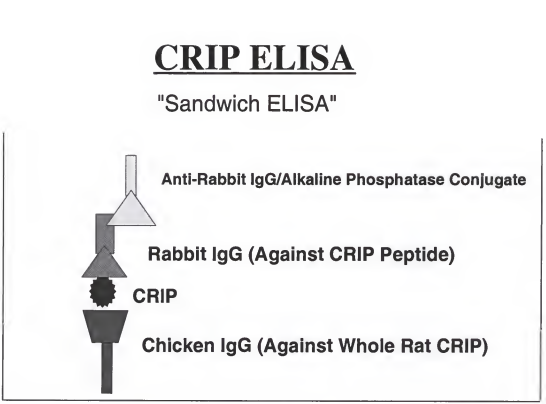


Figure 2-1:Schematic representation of CRIP Sandwich ELISA. The hydrazide coating of the microtiter plates ensures the orientation of the Chicken IgG as depicted here.

partners (α-actinin and the cysteine rich protein (CRP) (Crawford et al., 1994; Sadler et al., 1992). Beckerle's group at the University of Utah determined that this partnership requires one of zyxin's three LIM domains (Schmeichel and Beckerle, 1994) and that the LIM domain mediates protein-protein interactions.

Another LIM-only protein, LMO2 (previously Rbtn2 or Ttg2) functions as a bridge which assembles a protein complex. Different research groups pieced together the various proteins of the complex. When LMO2 dimerizes with the protein Tal1 in thymocytes of transgenic mice, thymocyte development is altered leading to T-cell tumorigenesis (Larson et al., 1996). Another protein-partner for LMO2 is nuclear LIM interactor (NLI, Jurata et al., 1996) which is also referred to as LIM domain binding protein (Ldb1, Agulnick et al., 1996). This protein was found to be important for neuronal development. Finally, Rabbitts group (Wadman et al., 1997) used eletrophoretic mobility shift assays and nuclear extracts to demonstrate the complete complex which includes LMO2, Tal1, E2A (from a class of proteins that bind E-box motifs which have the general sequence CANNTG), GATA-1 (first member of the family of zinc finger protiens that bind the DNA sequence GATA) and NLI (or Ldb1). The DNA-binding complex which forms plays a role in hematopoiesis (Wadman et al., 1997; Yamada et al., 1998).

Whether CRIP has the ability to bind to other proteins or DNA has been postulated and examined (Perez-Alvarado et al., 1996).

The solution structure of rat CRIP has been determined by nuclear magnetic resonance spectroscopy (Perez-Alvarado et al., 1996). Surface potential maps showed that there are patches of hydrophobic surface which are candidate sites for recognition by other proteins. The three-dimensional folds of CRIP form a substructure that may be capable of binding DNA. It may also be possible that CRIP may interact with both proteins and nucleic acids through the LIM domain. Whether CRIP binds nucleic acids or proteins might be related to the conformation of the LIM domain, thus modifying the affinity of the domain for DNA or protein targets. Determining the protein- or DNA-partners for CRIP will prove very useful in defining a function(s) for CRIP and the LIM motif in general.

Previous work completed in our lab has led to identification of potential protein-partners for CRIP. Using affinity chromatography and an overlay assay, CRIP was found to interact with albumin, trypsinogen, and possibly N-CAM (adhesion molecule) or nitric oxide synthase. As a group, these proteins are interesting in that they are associated with the inflammatory response. Future studies should be directed at these specific proteins and their interaction with CRIP. Although I have not focused on this particular research question in my project, the possibility of a protein-

partner for CRIP was evident in one series of experiments which I performed. I examined whether treatment of cytosolic preparations from mouse small intestine, spleen, and thymus with the detergent sodium dodecyl sulfate (SDS) affected my ability to quantify CRIP protein using the CRIP ELISA.

Materials and Methods

Antibodies Chicken immunoglobulin G (IgY) against recombinant rat CRIP (rrCRIP) was isolated using a method adapted from Gassman et al. (1990) and Hassal and Aspock (1988). After initially inoculating young laying hens with 100 μg of rrCRIP (Khoo and Cousins, 1993) suspended in complete Freund's adjuvant (Smith et al, 1992), the hens were boosted with additional injections of rrCRIP in incomplete Freund's adjuvant at 2 week intervals. The contents of each separated egg yolk were suspended in 60 ml of polyethylene glycol (4.4%) with shaking. After 30 min at room temperature, the mixture was centrifuged at 3600 x g for 1 h at 4°C. The supernatant was filtered (0.45μ) and placed on a Sepharose CL4B (Pharmacia, Piscataway, NJ) hydrophobic interaction column. The IgY was eluted from the column with dd H_2O. Proteins were identified in fractions by measuring the absorbance at 280 nm. The IgY fractions were pooled and concentrated by ultrafiltration. In addition, a rabbit immunoglobulin G (IgG) was raised against a rCRIP peptide (amino acid residues 64-77 with Cys

substituted for Lys at residue 64) basically as previously described (Fernandes et al., 1997).

Sandwich ELISA Carbohydrate binding microtiter plates (Corning Costar) were coated according to the manufacturer's directions. Briefly, chicken anti-CRIP IgY was diluted to 0.5 μg/ml in sodium acetate (10 mM, pH 5.5). The IgY solution was allowed to react with sodium periodate (3.2 mg/ml) IgY solution) for 30 min at room temperature, with gentle shaking. The activated antibody was then placed onto the microtiter plate (100 μl/well) and allowed to incubate for 1 h. The plate was rinsed 3 times with Tris-buffered saline/Tween-20 (TBS-T, 20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween-20). The remaining active sites were blocked with SuperBlock Blocking Buffer in TBS (Pierce, Rockford, Illinois) according to the manufacturer's instructions. The SuperBlock was decanted and the plates were used immediately or stored at -20°C.

Recombinant human CRIP (rhCRIP, Khoo et al., 1997) or rrCRIP was serially diluted with buffer (1% SuperBlock in TBS) in a range suitable for the unknowns (typically 0-4800 ng/ml). Serial dilutions of the intestinal cytosol were placed on the microtiter plate. The primary detection antibody was the rabbit IgG described above. A secondary alkaline phosphatase-labeled anti-rabbit IgG (Sigma) was used to detect the CRIP/antibody complex. P-nitrophenylphosphate (Pierce) diluted to 1 mg/ml in

diethanolamine substrate buffer (Pierce) was the substrate. CRIP concentrations were determined using regression analysis of the absorbance at 405 nm.

Modification of Sample Preparation Mouse small intestine was excised, the mucosal layer was removed, and a cytosol fraction was prepared as previously described (Hempe et al., 1991 and Levenson et al., 1993). A cytosol fraction was prepared in the same manner for spleen. Protein concentration of the cytosol was determined (Lowry et al., 1951). In a manner similar to that of Western analysis, the cytosolic preps were treated with SDS (final concentration 2.5%). A portion of the preparation was left untreated for comparison. The samples were then tested in the ELISA as described above. Additionally, SDS treatment was tested without the addition of a protein sample and with the rCRIP standard as controls.

Results/Discussion

CRIP ELISA Standard Curve Egg yolks from different chickens were used in the preparation of IgY in order to choose the antibodies with the maximal titer. Both chickens and rabbit antibodies were also tested for use as either the capture antibody or primary detection antibody (data not shown). After this evaluation, chicken IgY was selected as the capture antibody, and rabbit IgG was selected as the detection antibody. Specificity of the ELISA was evaluated by comparing pre-immune rabbit serum vs. anti-CRIP peptide

IgG as the detection antibody (Figure 2-2). Absorbance at 405 nm is directly proportional to the CRIP concentration, but only with the anti-CRIP IgG. There was a proportional absorbance increase when rrCRIP was added to test samples. Furthermore, other proteins do not produce significant absorbance (data not shown). The average standard curve (n = 9), produced a linear line which had a correlation coefficient of 0.98 (Figure 2-3). The interassay and intraassay coefficients of variation were 10.3% and 15.7%, respectively. In addition, specificity of the detection antibody was previously demonstrated by western analysis (Fernandes et al., 1997).

CRIP Protein CRIP was quantified in small intestinal and spleen samples using the ELISA (Table 2-1). This table depicts the CRIP protein levels of samples tested. When the small intestine samples were treated with SDS, there was essentially no change in the CRIP level or absorbance. However, when spleen samples were not treated with SDS, there was no dectectable CRIP in the samples and the absorbance did not rise above background. Thus treatment of the samples with SDS was effective in allowing CRIP to be quantified in a variety of tissue preparations. This modification was applied to all thymus and spleen samples used in the ELISA for the experiments described herein.

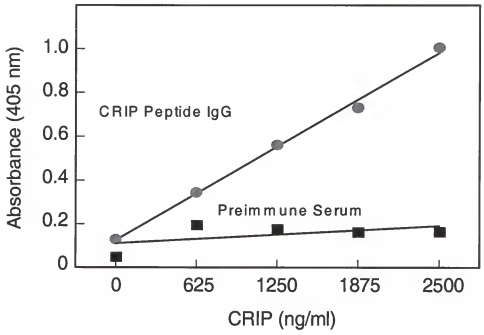


Figure 2-2. Standard curve for the detection of murine CRIP by sandwich ELISA. Chicken anti-rat CRIP IgY was used as the capture antibody and rabbit anti-CRIP peptide IgG was the detection antibody. Substitution of preimmune serum for the rabbit IgG is also shown. Values are the absorbance at 405 nm with dilutions of rrCRIP.

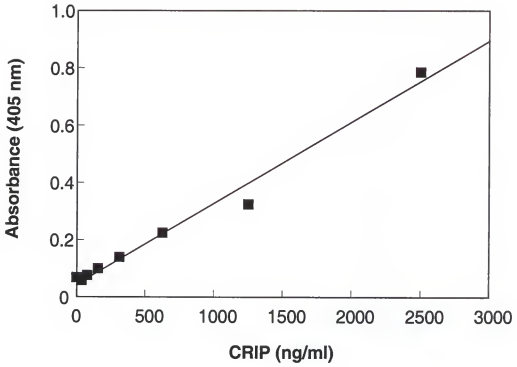


Figure 2-3. Average standard curve for the detection of CRIP by sandwich ELISA. Chicken anti-rat CRIP IgY was used as the capture antibody and rabbit anti-CRIP peptide IgG was the detection antiody. Values are the average of 9 standard curves. Regression analysis revealed a linear line which had a correlation coefficient (r^2) of 0.98. The interassay and intraassay coefficients of variation were 10.3% and 15.7%, respectively.

Table 2-1: Treatment of spleen and intestine samples with detergent.

Sample	Detergent (+/-)	CRIP (μg/g tissue)
Spleen	(-)	Not Detectable
Spleen	(+)	21.7
Intestine	(-)	21.9
Intestine	(+)	22.0

Values shown are means for a single experiment.

CHAPTER 3
HUMAN MILK MONONUCLEAR CELLS

Introduction

Human milk is an important immunological fluid which is beneficial to the newborn. Milk provides immunoglobulins (primarily as secretory IgA), lactoferrin, lysozyme, bifidus factors, complement factors, epidermal growth factor, and catalase (Hanson et al., 1988; Lawrence, 1994; Newman, 1995). Production of these factors is local because milk contains several cell types including macrophage, epithelial cells, lymphocytes (both B- and T-lymphocytes), and natural killer (NK) cells (Newman, 1995; Lawrence, 1994). Levels of these cells are highest in colostrum and decline markedly over the first 6-8 weeks of lactation. Recent research has demonstrated that the mononuclear cells (monocytes and lymphocytes) have the potential to produce several different cytokines (Skansen-Saphir et al., 1993). Thus, milk mononuclear cells are capable of being stimulated in the milk to produce important mediators of immune responses. I chose to examine this cell population for CRIP mRNA and protein.

If CRIP was found to be present in human milk, questions would arise as to its function in this fluid. It is possible that it may serve a local

function in the breast or it may be important for the newborn. Examining CRIP's pattern of expression during lactation would also be useful in understanding its function in human milk.

Materials and Methods

Milk Collection Subjects were recruited in the Shands at the University of Florida Mother/Baby Unit by the Unit's Lactation Coordinator. The women were selected initially on their ability to donate a one-time sample. In a later set of experiments, a group of women was selected on their ability to make several donations over the first 4-6 weeks of lactation. After obtaining informed consent, milk was collected semiaseptically by low-pressure pump (Medela Inc., McHenry, IL) into sterile polypropylene bottles. The milk was stored at 2-4°C for no more than 24 hrs before processing.

Milk Cell processing A portion of whole milk (1-2 ml) was retained to test for the presence of CRIP in the fluid. The total milk cells were isolated using a modified method of Saito et al. (1991). Briefly, the whole milk was centrifuged at 3000 x g for 30 min (2-4°C). After the fat layer and whey were set aside, the cells were resuspended in phosphate buffered saline (PBS). A portion of the whey was also retained for CRIP analysis. The MMNC, lymphocytes, and monocytes were obtained by centrifugation on density gradients (Histopaque; Sigma Chemical, St. Louis, MO, Nycoprep,

and Histoprep; Sigma, respectively) according to the manufacturer's directions.

CRIP ELISA A portion of MMNC was treated with protease inhibitors (2 mM phenylmethylsulfonyl fluoride, pepstatin 0.9 μg/ml, and leupeptin 0.6 μg/ml) and was lysed by sonication. The sonicated cells were centrifuged at 40,000 x g for 30 min at 4°C. The cytosol was collected for quantification of CRIP protein. Total protein content of the MMNC cytosol portion was determined (Lowry et al., 1951). The cytosolic fractions were then subjected to the CRIP ELISA (as described in Chapter 4, Materials and Methods).

Northern Analysis A separate portion of milk cells was used for the isolation of total RNA, which was extracted using TRIzol reagent (GIBCO-BRL, Gaithsburg, MD). Northern analysis was carried out on 10-20 μg of total RNA on 1% agarose/formaldehyde gels as described in Chapter 4, Materials and Methods). Human CRIP and 18S cDNA probes were prepared by RAD-Prime Labeling (GIBCO/BRL) with ^{32}P-dCTP. Densitometry was performed using Intelligent Quantifier 2.1 (BioImage, Ann Arbor, MI) with the 18S signal as the reference.

Flow Cytometry Human MMNC were collected and isolated as described above. After isolating the MMNC from the Histopaque gradient, the cells were washed thoroughly (4 times) in PBS. The cells were counted and

diluted to a concentration of 1 x 10^6 cells in staining medium (1% bovine serum albumin, 0.05% sodium azide in PBS). Fluorescein isothiocyanate (FITC)-labeled mouse anti-human CD14 (Chemicon International Inc., Temecula, CA) and phycoerythrin (PE)-labeled mouse IgG (Pharmingen, San Diego, CA) were used. Flow cytometry was performed using a Becton-Dickinson FACScan instrument and Lysis software.

Statistical Analysis Linear regression (Excel software, Microsoft, Redmond, WA) was used to determine CRIP concentrations. Tests for significance were performed using the InStat Biostatistics software package (GraphPad Software, San Diego, CA) or the SAS System (SAS Institute Inc., Cary, NC).

Results

CRIP mRNA CRIP mRNA expression was initially evaluated in subjects who were donating a one-time sample of milk. Milk mononuclear cells were isolated from milk collected on days 3, 4, 7, 21 and 28 postpartum. Total RNA isolated from IEC-6 (intestinal) cells which had been transfected with CRIP cDNA was used as a control sample (Figure 3-1). CRIP mRNA expression was highest for the transfected cells and CRIP mRNA expression in the milk cells was expressed relative to the IEC-6 cells. CRIP mRNA expression was highest on day 3 (43.2%) postpartum and fell over the first week (2.9% of control on day 7). By day 21 (9.2% of control), CRIP mRNA began to increase again and was back to approximately the day 4 level

(21.9% of control) by day 28 (23.4% of control). This group of experiments was important in that this was the first time that CRIP expression had been detected in human milk mononuclear cells.

CRIP Protein In order to confirm that the mRNA was being translated into protein, cells were collected for use in the in the CRIP sandwich ELISA (Chapter 2). For this group of experiments, cells were collected at 4, 5,7, 11, 14, 21, and 49 days postpartum from separate subjects (Figure 3-2). Samples collected on days 4, 7, 14, and 21 corresponded to the same samples from which CRIP mRNA was isolated from. When CRIP protein was quantified in these cells, rat intestinal cytosol was used as a standard. The human MMNC in general produced more CRIP protein as compared to the rat intestine. As with the mRNA expression, CRIP protein levels dropped to the lowest point on day 7 and then increased during the second week. On day 11, CRIP protein was the highest. Interestingly, CRIP protein levels were lower on day 21 for this subject (similar to what was observed with the mRNA). In order to understand why CRIP levels might be high or low on particular days (i.e. day 7 was low and day 11 was high), medical records for the subjects were obtained to rule out a possible effect of maternal infection or parity. This information was also evaluated by the lactation consultant. Looking at the subjects as a group, there were no unusual characteristics or signs of infection. The next step was to select subjects

who could donate milk on a regular basis for CRIP protein quantification in their MMNC.

Longitudinal Study Four subjects were recruited for these experiments. Milk was collected and MMNC were isolated on days ranging from 3-39 days postpartum (Figure 3-3). Milk was collected on an approximate weekly basis for each subject. CRIP protein levels in the milk cells fell into a range of 5-10 μg/mg of total cell protein at the beginning of the collection period and were in a range of 2-8 μg/ mg of total cell protein at the end of the collection period. In general, CRIP protein levels in the human MMNC fell over the collection period. When all of the data was analyzed from each subject for homogeneity of slopes, the correlation coefficient (r^2) was 0.927. Thus there was a strong correlation between CRIP protein in human MMNC and stage of lactation. There was a statistically significant difference ($P < 0.0004$) in the slopes of the lines from each subject when CRIP protein was compared to day postpartum. Therefore, the data from all subjects was not combined in order to determine the correlation coefficient.

Flow Cytometry The CD14 antibody is specific to monocytes (in this case to human monocytes). The isotype control is used as an estimation of non-specific staining. By comparing the FITC-labeled cells to the PE-labeled cells, an estimation of the number of monocytes in the milk samples collected 2 weeks postpartum could be obtained. In the human milk

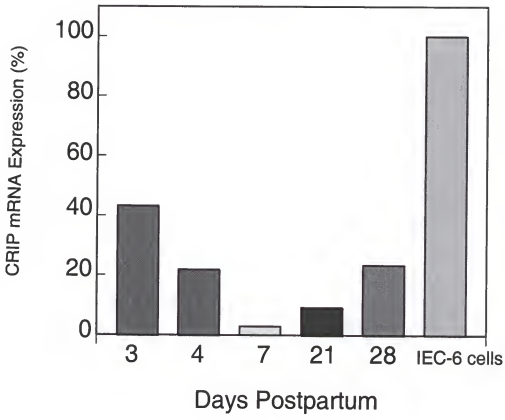


Figure 3-1. Comparison of CRIP mRNA levels in human milk mononuclear cells and CRIP-transfected IEC-6 cells. Total RNA extracted from these cells was used for northern analysis. rCRIP and 18S cDNAs were used as probes. Relative abundance of CRIP mRNA compared to that of 18S mRNA was measured by digitized densitometry of individual bands. CRIP mRNA expression in the milk cells is expressed relative to the IEC-6 cells. Values shown are from milk mononuclear cells collected on a single day from separate subjects.

mononuclear cell sample, 71% of the cells were expressing the CD14 surface marker and 19% of the cells were stained using the isotype control. Thus approximately 52% of the cells collected were monocytes.

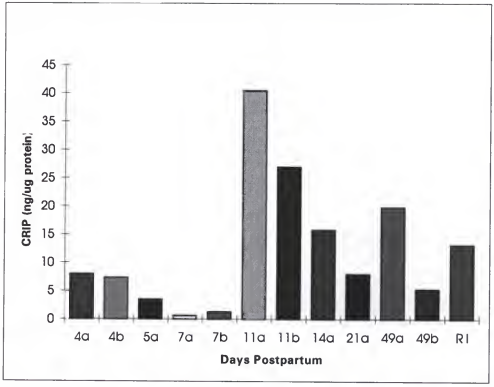


Figure 3-2. Comparison of CRIP protein in human milk mononuclear cells from individual subjects. Total cytosol protein was used from isolated mononuclear cells in the CRIP ELISA. Values are CRIP protein levels from a single subject. 4a, sample collected 4 days postpartum from subject "a"; 4b, sample collected 4 days postpartum from subject "b"; etc.

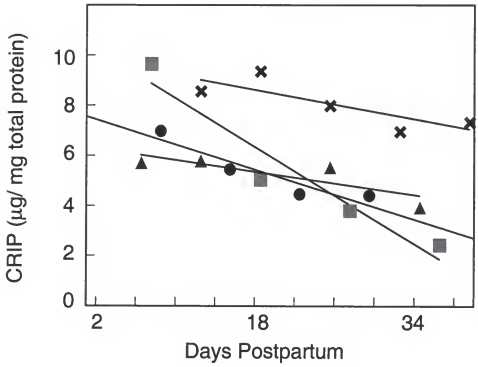


Figure 3-3. Comparison of CRIP protein in human milk mononuclear cells from 4 subjects. Total cytosol protein was used from isolated mononuclear cells in the CRIP ELISA. Each point represents the CRIP protein level from a single subject on a separate day.

Discussion

The American Academy of Pediatrics statement on Breastfeeding and the Use of Human Milk is supported by extensive research which demonstrates the advantages of breastfeeding for infants, mothers, families, and society (AAP, 1997). In this statement, it is suggested that breastfeeding continue for at least the first 12 months of life, and thereafter for as long as mutually desired. Increasing rates of breastfeeding is also one of the goals of Healthy People 2000. This target goal is to "increase to at least 75% the proportion of mothers who breastfeed their babies in the early postpartum period and to at least 50% the proportion who continue to breastfeed until their babies are at least 5 to 6 months old" (US Dept of Health and Human Services, 1990). Much of the attention which has been given to breastfeeding and human milk in the last decade is related to the fact that human milk provides several advantages to infants with regard to growth, general health, development, and reduction in both acute and chronic diseases. Thus understanding the factors/components of human milk which are responsible for these benefits is important.

In this group of experiments human milk was collected for the isolation of MMNC. These cells are capable of making several factors such as cytokines, hormones, and growth factors which are proposed to provide active immunity for the infant. Because there is evidence which supports a

role for CRIP in immune function, it was of interest to evaluate CRIP expression in these cells.

Human MMNC which were collected over the first couple of months of lactation were able to make both CRIP mRNA and protein. As mentioned in Chapter 1, the zinc content of breast milk during this period is tightly regulated, being affected very minimally if at all by maternal zinc status or infection. Because zinc is important for immune cell function, perhaps there is some relationship between the zinc content of milk and the immunological benefits of this fluid during the neonatal period.

In the human infant, the gastrointestinal tract is not fully functional during the first couple of months postpartum, and this is especially true for the premature infant. The term infant may experience decreased protease activity (Neu and Koldovsky, 1996; Hamosh, 1996), decreased gastric acidity (McNeish, 1984), and increased permeability of intestinal mucosa to forgein proteins (Mannick and Udall, 1996; Brandtzaeg, 1998; Insoft et al., 1996). Additionally, there are decreases in bile acid concentration and decreased motility. Collectively, these decreases in intestinal function, such as protease activity and permeability, may compromise local defense mechanisms of the intestine in premature and full-term infants.

There is evidence that several factors in milk may be protective for the infant during this period. For example, studies in mice demonstrate that

the transfer through milk of antibodies against viral and bacterial antigens can prime immune responses in offspring (Stein and Soderstrom, 1984). In humans, anti-idiotypic antibodies to polio virus have been found in milk (Hahn-Zoric et al., 1993) and it has been suggested that the ability to transfer these antibodies to the infant via milk may enhance the capacity of breastfed infants to respond to polio-virus vaccine (Hahn-Zoric et al., 1990). There are also studies which have shown that milk leukocytes can be taken up intact by offspring in mouse, rats, lambs, and baboons (Weiler et al., 1983; Sheldrake and Husband, 1985; Jain et al., 1989).

It has been proposed that the infant has increased tolerance to HLA on the maternal milk cells taken up via the gut of the breastfed infant (Hanson, 1998). This might allow the maternal milk lymphocytes to transfer immunologic information to the offspring without induction of an immune response to the cells. Thus the cells which contain CRIP may actually be taken up by the infant intact. This would fit well with the demonstration that intestinal CRIP expression is low prior to weaning (Birkenmeier and Gordon, 1986; Levenson et al., 1993).

In the infant gut, there may be a substantial ability to produce Th2 lymphocyte cytokines (Youwu et al., 1994) and a lesser ability for Th1 lymphocyte cytokines (Bocci et al., 1993). With a decrease in such cytokines as IFN-γ and an increased ability to make the anti-inflammatory

cytokines IL-4 and IL-10, the local immune repsonse may be somewhat suppressed. Since cytokines such as IFN-γ are produced in human milk (Hanson, 1998), this may help to stimulate mucosal immunity while the infant's own gut-associated lymphoid tissue (GALT) matures. If CRIP, like IFN-γ, is produced at lower levels in the human intestine (as with the rat), its presence in human milk may also serve to be benficial to the infant while the gut is continuing to mature. As will be demonstrated in later Chapters, CRIP expression may play some role in cytokine balance. In this capacity, CRIP's presence in milk may also aid in cytokine balance in the infant's mucosal immune system as it matures. Clearly, the presence of CRIP in human milk may prove to be important and should be a focus of future work with the protein.

CHAPTER 4
FURTHER CHARACTERIZATION OF CRIP TRANSGENIC MICE

Review of Initial Characterization

In order to gain more information on the function of CRIP, a line of transgenic mice which overexpress the rat CRIP gene was created (Davis et al., 1998a). These mice were created at the National Institute of Child Health and Human Development-supported Transgenic Mouse Development Facility at the University of Alabama-Birmingham using a transgene which was created in our lab. The transgene contained 1.75 kb of the rCRIP promoter region with 2.6 kb of the coding and downstream region plus 91 bp of a pGEM vector sequence. This vector sequence was crucial for later identification of the transgenic mice from littermates because there is a high degree of homology between rat and mouse CRIP cDNAs.

The transgenic founder mice were created via microinjection of the rat CRIP cDNA construct into C57BL/6J X SJL/J F2 hybrid mouse zygotes (Polites and Pinkert, 1994). The transgenic mice were brought back to the University of Florida and maintained under specific-pathogen free (SPF) conditions at the Transgenic Mouse facility. In this environment, extra precautions were taken to limit the spread of pathogens within the new

colony. The cages, water, and bedding were autoclaved. The mice were fed a rodent diet for breeders (Teklad 7902) which was gamma-irradiated. The mice were housed in microisolator cages (12x8x5 in.) which were only opened under laminar flow hoods.

Initial characterization of the CRIP-Tg mice was carried out over the next year. The founder mice were mated with C57BL/6J X SJL/J mice (The Jackson Laboratory, Bar Harbor, ME) and the heterozygous animals were identified for the presence of transgene as described below. The mice which overexpressed the CRIP transgene were then compared with their non-transgenic littermates for CRIP mRNA and protein expression in several tissues. Flow cytometry was used to analyze thymocyte populations in the transgenic mice and blood cell populations and other blood parameters were analyzed. Finally, immunohistochemical analysis was performed on selected tissues.

Both CRIP mRNA and protein levels were increased in CRIP-Tg mice as compared to NTg littermates. A comparison tissue survey of CRIP mRNA expression from CRIP-Tg and NTg mice was performed. As previously reported in the rat (Hallquist et al., 1996), CRIP mRNA expression was highest in the small intestine (duodenum), spleen, thymus, lung, prostate, and heart. CRIP mRNA expression was consistently elevated in transgenics as compared to non-transgenics. Increased protein levels were observed in

the intestine, spleen, and thymus of CRIP-Tg mice as compared to NTg mice (Figure 4-1). The magnitude of the increase in protein and mRNA was roughly equivalent.

Because there is constitutive high expression of CRIP in lymphoid tissue (Hallquist et al., 1996; Khoo et al., 1997; Fernandes et al., 1997), it has been proposed that this protein may have a function in immune cells. Thus, thymocyte populations and blood cells were examined to ascertain the effect(s) of the overexpression of this protein on certain cell types. Flow cytometry data suggested that CRIP-Tg mice have more $CD4^+/CD8^+$ thymic lymphocytes. Analysis of blood parameters (Table 4-1) demonstrated that although most values such as hemtocrit or red blood cells were within the normal range and not different between groups, the CRIP-Tg mice had approximately half of the white blood cell count found in the NTg mice. There was also a decrease in the percentage of lymphocytes and an increase in the percentage of monocytes, eosinophils, and neutrophils.

Immunohistochemical analysis was performed on selected tissues to localize CRIP protein in these tissues and to evaluate whether any morphological changes occurred when the CRIP protein was overexpressed (Davis et al., 1998a). There were no overt morphological changes in intestine, thymus, popliteal lymph node, spleen, or peritoneal macrophage as observed by staining with hematoxylin and eosin (H&E). CRIP protein

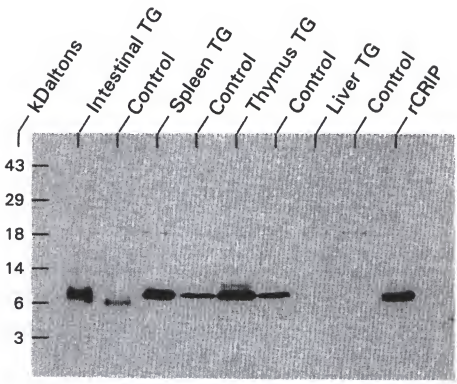


Figure 4-1. Relative abundance of CRIP as measured by Western analysis. Cytosol fractions from each tissue of transgenic and nontransgenic control mice were separated by 15% Tris-Tricine SDS-PAGE and transferred to nitrocellulose membranes. Western analysis utilized affinity-purified rabbit anti-CRIP peptide IgG and enhanced chemiluminescence. Purified recombinant rCRIP was used as the standard.

was localized to the mucosa, submucosa, lamina propria, and villus epithelial cells in the small intestine. In the thymus, CRIP was localized to the medulla as compared to the cortex of this lymphoid organ. CRIP was present in the outer cortex of the popliteal lymph node and in the marginal zones in the white pulp of spleen. Additionally, CRIP was localized to the cytoplasm of peritoneal macrophage.

Table 4-1. Leukocytes in blood from Non-Tg and CRIP-Tg mice.

Genotype/ Environment	White Blood Cells (K/μL)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Neutrophils (%)
SPF					
Non-Tg	2.7 ± 0.4	82.4 ± 1.2	3.6 ± 0.9	1.2 ± 0.7	12.8 ± 4.4
CRIP-Tg	1.5 ± 0.4	64.1 ± 2.8	9.1 ± 3.0	5.7 ± 1.2	21.0 ± 4.3
P value	0.05	0.0003	N.S.	0.007	N.S.

Values shown are mean ± SEM, n = 4-7 mice each value
N.S. = Not Significant.

Taken together,the data described above provided much of the initial information which we needed to decide how to utilize our newly-developed transgenic model. However, before work could begin with the model, we had to develop a breeding strategy in order to achieve homozygous animals. The initial characterization of this line of transgenic mice was performed using heterozygous mice. Additionally, basic questions about phenotypic

outcomes needed to be answered. After the homozygous line was established, measurements of body and organ weights were obtained. With these data collected, experiments were designed to utilize the model.

Breeding Strategy

As outlined above, the founder mice were produced via microinjection of C57BL/6J X SJL/J F2 hybrid mouse zygotes. Upon arrival to the University of Florida, the founders were mated with C57BL/6J X SJL/J F1 mice to establish transgenic lines. These breeding pairs were considered the F0 generation for the transgenic line. Offspring (F1) from this cross were examined for transgene and estimation of copy number. The F1 offspring are typically used as colony founders in order to eliminate the possibility that the mice are chimeric. Chimerism could result if the transgene is not integrated into the genome of every cell. In addition, the use of F1 offspring for founders eliminates the problem of multiple integration sites and numbers which may be present in the F0 generation.

Thus from the initial mating of the transgenic founders with hybrid mice, the resulting F1 generation was hemizygous for the transgene (approximately fifty percent were transgenic). Presence of the transgene was determined using polymerase chain reaction (PCR, Davis et al., 1998a). Briefly, genomic tail DNA was isolated (Gordon, 1993) from mice at weaning (approximately 3 weeks of age). Primers were selected in order to

amplify a 342-bp region at the 3' end of the rCRIP transgene and the downstream primer was complimentary to the pGEM vector tail. This allowed for the amplification of the transgene only, excluding the amplification of the endogenous mouse CRIP gene.

After identification of transgenic mice, the copy number was then determined. A slot-blot technique was used to determine how many copies of the transgene were present in the offspring. The genomic tail DNA (20 μg) was blotted onto a nitrocellulose membrane. On this same membrane, a standard curve was created with 0, 1, 5, 10, 15, and 20 transgene copies/genome in genomic DNA. The membrane was then UV crosslinked and hybridized (overnight, 65°C) with a labeled 359-bp rat CRIP cDNA (Levenson et al., 1993). The cDNA was labeled with ^{32}P-dCTP (NEN/DuPont) using the Random Primer Labeling System (GIBCO/BRL, Gaithersburg, MD) as previously described (Khoo et al., 1997). After washing, the membrane was exposed to X-ray film (Figure 4-2). A mouse ß-actin cDNA was labeled as above and used as the normalization standard for densitometry. Autoradiographs were digitized and densitometry was performed using Intelligent Quantifier 2.1 (BioImage, Ann Arbor, MI). The genomic DNA hybridization intensity was comapred to the hybridization intensity of the standard curve in order to estimate the copy number.

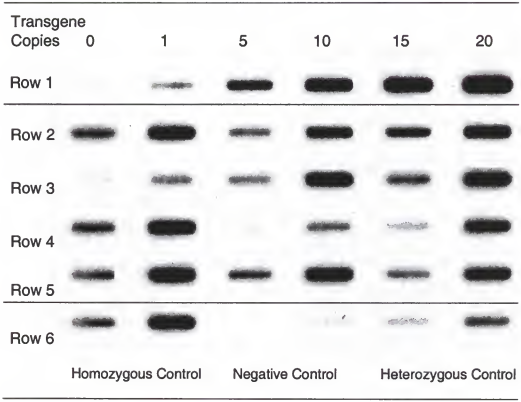


Figure 4-2. Slot blot technique for determining transgene copy number in CRIP transgenic mice. Genomic tail DNA (20 μg) was blotted onto nitrocellulose membrane along with a standard curve of 0, 1, 5,10, 15, and 20 transgene copies/genome in genomic DNA. The membrane was then probed with a ^{32}P-labeled 359-bp rat CRIP cDNA. Row 1 contains the standard curve with transgene copies as indicated. Row 6 contains Homozygous control, negative control, and heterozygous control. Unknowns were then compared to the standard curve and controls. For example, the first two slots on Row 3 were determined to be from a heterozygous mouse. The last 4 slots in this same row were determined to be from homozygous mice. There were no nontransgenic mice identified from this particular slot blot (other than negative control).

A slight modification of this identification/copy number estimation process made screening large numbers of mice more efficient. Because the standard curve for determining copy number also contained a "0 copies of transgene" point, which reflects murine CRIP expression rather than rat CRIP from the transgene, the animals could be screened for transgene and copy number in one assay, thus eliminating the PCR step. The usefulness of this screening process was especially apparent when we began breeding F1 siblings in order to obtain F2 offspring which were homozygous for the transgene. For example, the homozygous animals had approximately twice the number of transgene copies as the hemizygous animals and the non-transgenics did not have any transgene. Homozygous littermates were then bred (producing F3 offspring) in order to maintain the transgenic line. The transgenics were periodically screened for transgene copy number. A group of the homozygous transgenics were kept in the SPF facility and a group was brought to the Food Science and Human Nutrition Department mouse facility (a conventional housing facility).

Body and Organ Weight Analysis

Body and organ weights were obtained from transgenc and non-transgenic mice raised in either the SPF facility or the conventional facility. Organs weighed included spleen, thymus and liver. Spleen and thymus represent tissues in which there is an abundance of CRIP mRNA and protein,

whearas there is very little in liver. The mice were age and sex matched and measurements were recorded when the mice were approximately 4-12 weeks of age.

Results

Six founder mice were brought to the University of Florida for breeding purposes. After mating these mice with non-transgenic mice, four litters were obtained which had offspring that carried the transgene. From these four litters, screening was further carried out by comparing CRIP mRNA and protein expression. This comparison led to the selection of a single line which overexpressed CRIP mRNA at the highest levels in a tissue survey and was designated line 3626. This line also had the most copies of the CRIP transgene (approximately 5, heterozygous, Figure 4-2). The early characterization described above was carried out on this line of transgenic mice.

After mating F1 animals which carried the transgene (both female and male), the expected ratio of offspring was obtained. Medelian genetics dictates that a gene will be present in the offspring in a ratio of 1:2:1 (non-carriers:heterozygous:homozygous). This pattern was apparent on the slot blot in which animals were screened and copy number was determined. As pointed out above, the heterozygous mice had approximately 5 copies of the transgene, whearas the homozygous animals had approximately 8

copies of the transgene (Figure 4-2). The F2 offspring which were determined to be homozygous were then mated and all of the offspring from this cross (F3s) were homozygous as well. As a control for the identification/copy number determination method, hemizygous F2 animals were also mated. The expected ratio of 1:2:1 mentioned previously was obtained. Another control was using genomic mouse DNA from mouse tail biopsies from non-transgenic mice. There was consistently zero copies of transgene in these mice.

After establishing colonies of homozygous mice in both the SPF and conventional environments, body and organ weight analysis was carried out on the mice (Table 4-2). No statistically significant differences were observed for the parameters measured.

Table 4-2. Body and organ weights of CRIP-Tg and Non-Tg mice raised in different environments.

Parameter Environment/ Genotype	Body Weight (g)	Liver (mg/g BW)	Spleen (mg/g BW)	Thymus (mg/g BW)
SPF				
Non-Tg	26.5 ± 3.1	51.7 ± 5.1	4.1 ± 1.0	1.6 ± 0.5
CRIP-Tg	24.5 ± 3.2	47.3 ± 4.9	3.7 ± 0.8	1.6± 0.8
CONV				
Non-Tg	23.4 ± 3.4	48.5 ± 3.3	3.6 ± 0.6	1.8 ± 0.8
CRIP-Tg	25.7 ± 3.8	57.5 ± 4.6	4.3 ± 0.6	1.9 ± 0.5

Values shown are mean ± SD, n=9-12 mice each value.
SPF, specific pathogen free; CONV, conventional; Non-Tg, nontransgenic control mice; CRIP-Tg, CRIP overexpressing transgenic mice.

Discussion

The initial characterization and breeding strategy were crucial components of this project. In the development of a transgenic line or any colony of mice, it is important to have diligent maintenance practices which ensure that genetic quality is satifactory. These maintenance practices include proper identification, good husbandry techniques, keeping pedigrees, and alertness to phenotypic changes in the mice. These were the practices included in the development of our CRIP transgenic model.

The CRIP Tg mice were bred to the heterozygous stage for initial characterization. This initial analysis demonstrated that the selected line of mice was overexpressing both CRIP mRNA and protein, and especially in

intestine, spleen, thymus, and lung. It is interesting that CRIP overexpression remained highest in immune cells and tissues.

Immunohistochemical analysis pointed toward localization of CRIP in intestine, spleen, thymus, and popliteal lymph node (Davis et al., 1998). The staining pattern observed in the small intestine of the mouse was interesting when compared to earlier immunolocalization data in the rat small intestine (Fernandes et al., 1997). In the rat, CRIP was localized to the Paneth cell. The Paneth cell has been implicated as an effector of mucosal barrier function with its ability to secrete lysozyme, cryptidins, and secretory phospholipase A_2 (Ouellette, 1997). Although Paneth cells are strongly associated with innate immune function, dietary factors and luminal bacteria are not crucial to the establishment of the cell lineage. However, it is possible that these factors may explain the differences in immunolocalization of CRIP in the small intestine between the mouse and rat. The rats examined in the earlier work were raised in conventional housing, and the mice were raised in the SPF environment. This difference became the starting point of one of the main questions about CRIP: Is CRIP expression (especially in the intestine) affected by the environment in which an organism lives? This topic was further explored and is the subject of Chapter 5.

Other crucial aspects of the initial characterization was the examination of changes in certain physiological parameters associated with overexpression of CRIP. Flow cytometry data evaluating thymic lymphocyte populations further strengthened a role for CRIP in cellular differentiation, and in this case, cellular differentiation in an immune tissue (Davis et al., 1998a). CRIP overexpression led to an increase in the less mature double-positive ($CD4^+/CD8^+$) thymocytes. The T-cells of the double-positive immunophenotype express only low levels of the T-cell receptor, and if they cannot recognize the self major histocompatibility complex, they undergo apoptosis (Janeway and Travers, 1996). Another physiological change in the mice which overexpressed CRIP was a decrease in the total number of white blood cells and a decrease in the percentage of lymphocytes. These results suggest that CRIP is involved in control of cellular differentiation as there are fewer mature thymic T-cells and a decrease in the number of circulating white blood cells in CRIP Tg mice. These findings are consistent with the functions of other LIM-only proteins such as LMO1, MLP, and LMO2 as discussed in Chapter 1. The physiological significance of the lymphopenia produced by CRIP overexpression is also the focus of another group of studies in which T-cell related immune function is examined (Chapter 5).

CHAPTER 5
SENSITIVITY OF CRIP TRANSGENIC MICE TO ENVIRONMENT AND ENDOTOXIN

Introduction

CRIP expression is upregulated in the intestine of young rats just prior to weaning (Birkenmeir and Gordon, 1986; Levenson et al., 1993). The basis for this developmental increase follows a time course similar to the increase in some intestinal enzymes, which are inducted by glucocorticoid hormones. It was proposed that CRIP expression follows a comparable scenario of hormonal induction (Levenson et al., 1993) Indeed, the CRIP promoter has consensus sequences for glucocorticoid response elements and glucocorticoid hormone will induce CRIP in newborn mice (Levenson et al., 1993; Levenson et al, 1994a). Consequently, glucocorticoid hormones may contribute in part to the postnatal increase in expression.

However, since CRIP is highly expressed in immune tissues, we reasoned that this increase could also relate to exposure to dietary components and enteric microbial flora. The time course of CRIP expression prior to weaning approximates microbial colonization of the gastrointestinal tract (Bocci, 1992; Savage, 1986). Therefore, we decided to compare

CRIP expression in CRIP transgenic adult mice which were maintained in the SPF environment with those from a conventional environment. We also evaluated the sensitivity of these mice to a lipopolysaccharide (LPS) injection. In rats, CRIP mRNA and protein were increased in peritoneal macrophage and PBMC after an LPS injection (Hallquist et al., 1996). In these studies I demonstrated that CRIP levels are different in CRIP transgenic and non-transgenic mice raised in separate environments, and that CRIP overexpressing mice are more sensitive to an endotoxic shock. Both findings are consistent with a function for CRIP in host immunity.

Materials and Methods

Animals and Experimental Design All animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee. Mice used in this study were born in the University of Florida Specific Pathogen Free (SPF) Mouse Facility. They were allowed free access to autoclaved, deionized water and irradiated commercial diet (Teklad Rodent Diet 8604; Harlan, Madison, WI). At weaning, groups of CRIP-Tg or Non-Tg mice were moved to conventional housing or remained in the SPF facility. In the conventional housing conditions, the drinking water was not autoclaved, and the diet (Teklad 8604) was not irradiated. The mice were maintained in these environments for 4-8 weeks. Body and organ weight

analysis was conducted on mice from both environments. In one series of experiments, CRIP-Tg and Non-Tg mice which were born in and lived in the conventional environment were injected i.p. with LPS (*E. coli* 0127:B8;Sigma, 20 or 2 mg/kg body weight) or saline. One week after the injection, the surviving mice were killed for serum zinc analysis. A separate group of mice were killed at 24 h after the LPS or saline injections for serum zinc analysis as a measure of the LPS response. A group of mice were also injected with LPS (10 mg/kg body weight) and killed 24 hours later for serum cytokine analysis (see below). Tissue samples were collected as described below. The mice were age- and sex-matched for all experiments.

Tissue Collection and Processing At the end of the comparison period, the animals were anesthetized with methoxyflurane and killed by cardiac puncture. Intestine, spleen, and thymus were taken for both protein and mRNA analysis. In order to extract total RNA, a section of small intestine (1 cm of proximal duodenum), spleen, or thymus were homogenized with a Polytron (Brinkman Instruments, Westbury, NY) in TRIzol (GibcoBRL, Gaithersburg, MD). The mucosal layer was removed from the remainder of the small intestine, and a cytosol fraction was prepared as previosly described (Hempe et al., 1991 and Levenson et al., 1993). A cytosol fraction was prepared in a similar manner for spleen and thymus. Protein concentration of the cytosol was determined (Lowry et al., 1951).

Northern Analysis Northern analysis was performed as previously described (Hallquist et al., 1996 and Blanchard and Cousins, 1996) with 10-20 μg of total RNA from intestine, spleen, or thymus.

Sandwich ELISA Recombinant rat CRIP (rrCRIP) was serially diluted with buffer (1% SuperBlock in TBS) in a range suitable for the unknowns (0-2500 ng/ml). Serial dilutions of the intestine, spleen, or thymus cytosol were placed on the microtiter plate. The ELISA was performed as described in Chapter 2.

Blood Analysis Blood was collected in EDTA-containing tubes (Microtainer, Becton Dickinson, Franklin Lakes, NJ). Complete blood chemistries (CBCs) were performed on the same day in the Clinical Pathology Laboratory of the University of Florida College of Veterinary Medicine. Blood samples were analyzed on an Abbott/Cell-Dyn 3500 automated system. A portion of the blood was used for manual differential and morphologic evaluation. Serum was analyzed for zinc using atomic absorption spectrophotometry. Serum IL-10, IL-6, TNF-α, and IFN-γ production after LPS challenge was quantified using a commercial ELISA kit (Cytimmune, College Park, MD).

Statistical Analysis Linear regression (Excel software, Microsoft, Redmond, WA) was used to determine CRIP concentrations. Tests for significance were performed using the Student's t-test and 2x2 factorial ANOVA. The InStat Biostatistics software package (GraphPad Software, San Diego, CA)

or the SAS System (SAS Institute Inc., Cary, NC) was used for determining level of significance. When necessary, data were subjected to $\log_{10}$ transformation to lessed heterogeneity of variances.

Results

CRIP Protein CRIP concentrations in the cytosol fraction from intestine are shown in Figure 5-1. There is a 3.8-fold difference ($P < 0.0001$) between Non-Tg mice reared in SPF conditions vs. the conventional environment. There is also a significant difference ($P < 0.0001$) of 3.3-fold in these concentrations in the CRIP-Tg mice when maintained under the different environmental conditions. The additional eight copies of the rCRIP gene in the CRIP-Tg mice produced a slight but not significant ($P < 0.08$) increase in intestinal CRIP concentrations compared to Non-Tg mice in both environments.

As shown in Figure 5-2A and Figure 5-2B, the CRIP-Tg mice have higher levels of expression ($P < 0.05$) in spleen and thymus, respectively, than the Non-Tg controls. There is greater expression in the thymus of CRIP-Tg mice raised under conventional conditions than SPF conditions ($P < 0.05$). This difference is far less than observed in the intestine (Figure 5-1), suggesting the stimulus for the effect of environment in intestinal CRIP expression is enteral in origin. There was no significant effect of environment on CRIP expression in the spleen.

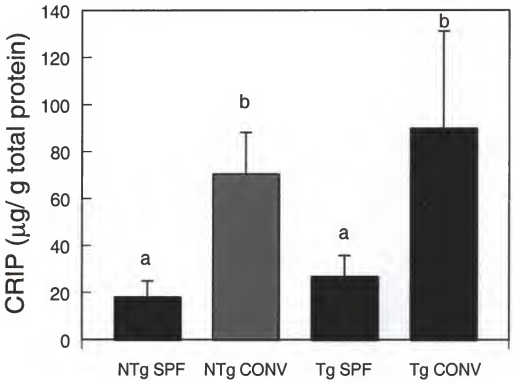


Figure 5-1. Comparison of intestinal CRIP protein concentrations in transgenic and nontransgenic control mice raised in specific pathogen free (SPF) or conventional (CONV) environments. Intestinal cytosol was prepared and CRIP was measured by ELISA. NTg, nontransgenic control mice; Tg, CRIP overexpressing transgenic mice. Values are mean ± SD, n=9-10 mice each value. Means with a different superscript are significantly ($P < 0.0001$) different.

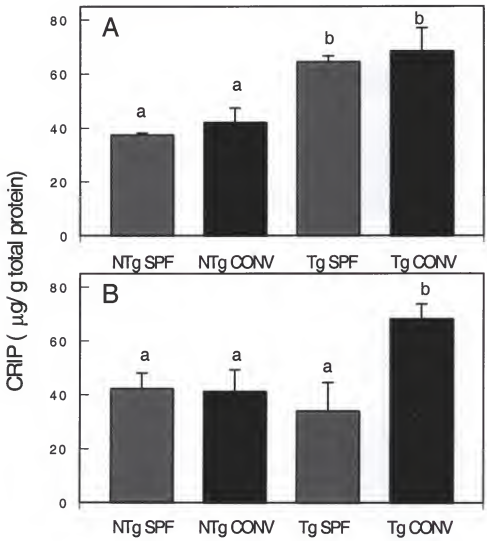
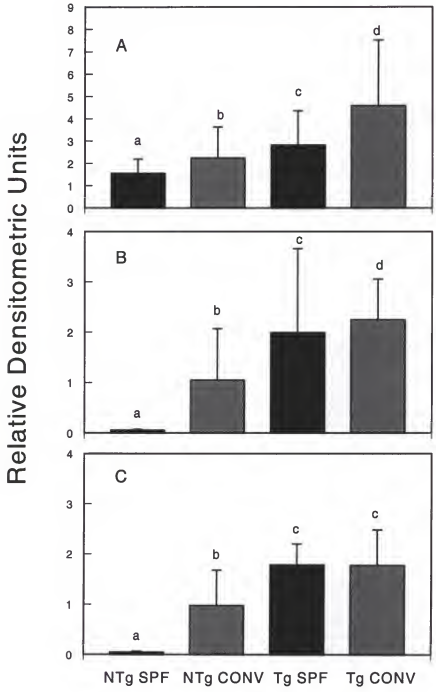


Figure 5-2. Comparison of CRIP protein concentrations in spleen and thymus of transgenic and nontransgenic control mice raised in specific pathogen free (SPF) or conventional (CONV) environments. Cytosol from spleen and thymus was prepared and CRIP was measured by ELISA. NTg, nontransgenic control mice; Tg CRIP overexpressing transgenic mice A: Spleen and B: Thymus. Values are mean ± SD, n=4 mice each value. Means with a different subscript are significantly ($P < 0.05$) different.

Figure 5-3. Comparison of CRIP mRNA levels in intestinal, spleen, and thymus of transgenic and nontransgenic control mice raised in specific pathogen-free (SPF) or conventional (CONV) environments. Total RNA extracted was used for northern analysis. rCRIP and β-actin were used as probes. NTg, nontransgenic control mice; Tg, CRIP overexpressing transgenic mice. Northern analysis of RNA from intestine (A), spleen (B), thymus (C). Relative abundance of CRIP mRNA compared to that of β-actin mRNA was measured by digitized densitometry of individual bands. Values are mean ± SD, n= 4-10 mice each value. Means with a different superscript are significantly different (intestine, $P < 0.05$; spleen $P < 0.0002$; thymus $P < 0.004$). Data were $\log_{10}$ transformed for statistical purposes as described in the text.


Relative Densitometric Units
A
B
C
a
b
c
d
NTg SPF
NTg CONV
Tg SPF
Tg CONV

CRIP mRNA Northern analysis showed that intestinal CRIP mRNA levels in the CRIP-Tg mice were significantly higher ($P < 0.007$) than the Non-Tg mice (Figure 5-3A). There was a significant increase ($P < 0.05$) in intestinal CRIP mRNA levels in both Tg and Non-Tg mice raised in conventional housing as compared to mice raised in the SPF environment.

Differences in CRIP mRNA expression in spleen and thymus between the two genotypes and two environments were markedly apparent from what was observed in the small intestine (Figure 5-3B and Figure 5-3C). There was a significant increase in expression ($P < 0.0001$) in the spleen or thymus of Non-Tg vs. Tg mice. Furthermore, there was a very significant effect of environment ($P < 0.0002$) on CRIP mRNA expression in the spleens of both CRIP-Tg and Non-Tg mice. The pattern of CRIP expression observed in the spleen was very similar to that of the thymus.

These results clearly show differences in CRIP expression in thymus and spleen compared to the intestine. For example, in intestine, CRIP mRNA and protein levels were strongly influenced by environment, whereas in the spleen and thymus CRIP mRNA was more strongly influenced by environment and protein was influenced to a lesser extent, if any. This differential pattern suggests that the small intestine is one of the main organs influenced by environmental factors, leading to a marked change in CRIP expression in this tissue. Since protein levels were not very different

in the spleen and thymus in response to environment as compared to changes in mRNA, a translational or postranslational regulatory step is likely to be responsible for the difference. Furthermore, the increases in CRIP mRNA in spleen and thymus suggest a systemic mediator derived from reponses of the intestine to environmental conditions is the mediator of differential CRIP expression in these tissues. In addition, CRIP is a zinc-requiring protein and the availability of this mineral in different tissues may be a factor in the regulation of this protein. Although intestinal CRIP levels appear to be more modulated by environment in the intestine than in the spleen or thymus, there was still somewhat of an effect in the thymus. This suggests that systemic host defense mediators, which are secreted in response to conventional husbandry conditions, have tissue specific effects on expression of the CRIP gene.

Complete blood counts The majority of blood parameters evaluated were similar among the groups of animals (Table 5-1). There was no significant difference in hematocrit, red blood cells, hemoglobin, or platelets, and these data were within normal ranges (data not shown). However, there was a highly significant decrease in the percentage of lymphocytes in the CRIP transgenic mice raised in the SPF environment ($P < 0.0003$). There was also a significant decrease in the total white blood cells (WBC) of CRIP-Tg mice raised in the SPF facility ($P < 0.05$). This decrease in lymphocytes

Table 5-1. Leukocytes in blood from Non-Tg and CRIP-Tg mice raised in different environments.

Genotype/ Environment	White Blood Cells (K/μL)	Lymphocytes (%)	Monocytes (%)	Eosinophils (%)	Neutrophils (%)
SPF					
Non-Tg	2.7±0.4	82.4±1.2	3.6±0.9	1.2±0.7	12.8±4.4
CRIP-Tg	1.5±0.4	64.1±2.8	9.1±3.0	5.7±1.2	21.0±4.3
P value	0.05	0.0003	N.S.	0.007	N.S.
CONV					
Non-Tg	1.9±0.6	79.8±3.2	5.4±2.3	1.4±0.4	13.4±3.1
CRIP-Tg	2.7±0.4	80.8±3.1	1.5±0.6	2.5±0.3	15.3±3.1
P value	N.S.	N.S.	N.S.	N.S.	N.S.

Values shown are mean ± SEM, n=4-7 mice each value
N.S., Not Significant

was accompanied by a statistically significant increase in the percentage of eosinophils ($P < 0.007$) in SPF CRIP-Tg mice as compared to all other groups. Although it was not statistically significant ($P > 0.05$), there was a trend toward an increase in the percentage of monocytes and neutrophils in the SPF CRIP-Tg mice. There was no difference in the total WBC between the two genotypes raised under conventional conditions. These data were obtained from 3 experiments which support the validity of the differences reported.

Organ/Body Weight Analysis There was not significant differences between genotypes or between environments for body weight and organ weights (data not shown, but very similar to Table 4-2).

LPS challenge studies Since the environmental differences observed in CRIP expression could most likely be explained by a response to microbial factors, we subjected these mice to an LPS challenge. Serum zinc was significantly decreased ($P < 0.001$) at 24 h in mice injected with LPS (Table 5-2). Reduced serum zinc concentrations are a well-recognized parameter of acute endotoxemia and demonstrate here the comparable response to LPS in both strains of mice. Following the LPS challenge, most of the CRIP-Tg mice died (Figure 5-4A). In contrast, only 25% of the Non-Tg mice died. During the course of the challenge, all Tg mice developed diarrhea (Figure 5-4B). They were lethargic and were not grooming themselves, which are features of endotoxic shock (Lowell et al., 1998). In comparison, only 50% of the Non-Tg mice developed diarrhea, and fewer of these mice appeared lethargic. The Tg mice lost significantly more weight ($P < 0.01$) by d 2 (Figure 5-4C). After this point, all mice began to recover from the LPS based on increased weight. At the end of the eight day challenge period, the transgenics still had a significantly lower body weight. The saline-injected mice did not lose weight, and none of these mice died (Figure 5-4C, 5-4A).

Table 5-2. Serum zinc concentrations in Non-Tg and CRIP-Tg mice treated with lipopolysaccharide (LPS).

Genotype	Treatment	Serum Zn (μg/ml)
Non-Tg	Saline	0.8 ± 0.1
Non-Tg	LPS	0.2 ± 0.1*
CRIP-Tg	Saline	0.9 ± 0.1
CRIP-Tg	LPS	0.2 ± 0.1*

Values shown are mean ± SEM, n = 3-5 mice each value. Mice were injected with LPS from *E. coli* i.p. (20 mg/kg) or saline. The mice were killed at 24 hr for serum zinc concentration.

The induction of CRIP intestinal expression in the two strains of mice was compared in the conventional environment, albeit 24 h after a lower LPS dose (2 mg/kg given i.p.) The rationale was that at this dose, which is not lethal, more primary effects attributable to LPS via secondary mediators would involve CRIP gene regulation. These mice were born and raised solely in the conventional environment, which differs slightly from the "conventional" environment denoted earlier where mice were born in the SPF environment and then moved to the conventional environment. As shown in Figure 5-5, CRIP protein levels are significantly higher ($P < 0.01$) in the LPS-treated transgenic mice. Clearly, this three- to fourfold higher CRIP level is correlated to the higher incidence of LPS-induced diarrhea in the Tg mice given the large LPS challenge (Figure 5-4B).

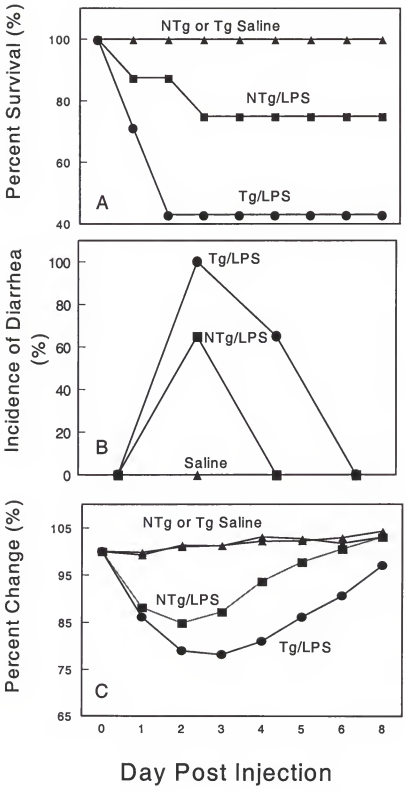
After mice were challenged with the lower dose of LPS, serum cytokines were measured (Table 5-3). Serum concentrations of IFN-γ were reduced nearly by half in the CRIP-Tg mice. This reduction was highly significant ($P < 0.005$). There were also very marked increases in serum of LPS-challenged CRIP-Tg mice for IL-10 and IL-6 ($P < 0.003$ and 0.03, respectively). The levels of IL-10 in serum were approximately 3 fold higher in CRIP-Tg as compared to Non-Tg. Serum IL-6 levels were 2 fold higher in the CRIP-Tg mice. There was not a significant change in serum TNF-α production between the two genotypes, although these levels were decreased by approximately 50% in the CRIP-Tg mice. Additionally, saline-treated animals were not producing detectable amounts of these cytokines (data not shown). Thus, stimulation of the immune system was essential for detectable differences in cytokine production.

Table 5-3. Serum cytokine concentrations in transgenic and nontransgenic control mice treated with lipopolysaccharide (LPS).

Cytokine	IFN-γ (ng/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)
Genotype				
Non-Tg	65 ± 11	756 ± 130	908 ± 422	173 ± 40
CRIP-Tg	24 ± 5	2082 ± 332	1211 ± 511	90 ± 24
P value	0.005	0.003	0.03	N.S.

Values shown are mean ± SEM, n = 6-8 mice each value. Mice were injected with LPS from *E. coli* i.p. (10 mg/kg) or saline. The mice were killed at 24 hr for serum collection. N.S., not significant

Figure 5-4. CRIP overexpressing mice have a greater response to LPS challenge. The mice were raised in a conventional environment. The mice were injected with LPS from *E. coli* i.p.(20 mg/kg) or saline, and were monitored over the subsequent 8 days. NTg, nontransgenic control mice; Tg, CRIP overexpressing transgenic mice. A. Mortality; B. Diarrhea incidence; C. Change from initial body weight. Data were derived from groups of n = 7-8 mice each value.


Percent Survival (%)
NTg or Tg Saline
NTg/LPS
Tg/LPS
A
100
80
60
40
Incidence of Diarrhea (%)
Tg/LPS
NTg/LPS
Saline
B
100
80
60
40
20
0
Percent Change (%)
NTg or Tg Saline
NTg/LPS
Tg/LPS
C
105
95
85
75
65
0
1
2
3
4
5
6
8
Day Post Injection

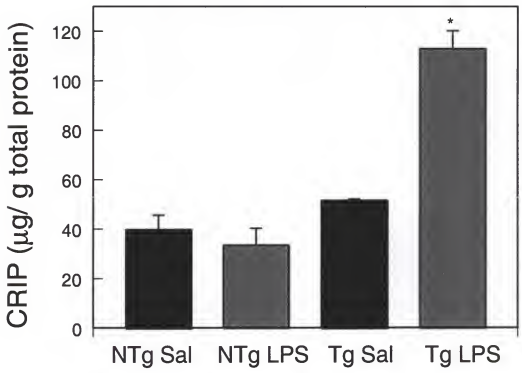


Figure 5-5. Comparison of CRIP levels in the intestine of transgenic and nontransgenic control mice treated with LPS from *E. coli* i.p.(2 mg/kg) or saline. The mice were raised in a conventional environment. CRIP concentrations were measured by ELISA. NTg, nontransgenic control mice; Tg, CRIP overexpressing transgenic mice. Values are mean ± SD, n=3-5 mice each value. *Means are significantly different ($P < 0.01$) from all other means.

Discussion

In this study, we have examined the expression of CRIP in both CRIP-Tg and Non-Tg mice which were raised in either SPF or conventional environments. We chose to examine CRIP expression and other parameters in these separate environments due to the upregulation of CRIP expression in the intestine of rats just prior to weaning (Birkenmeir and Gordon, 1986 and Levenson et al., 1993). This time period corresponds to the point at which the gut is adapting to both colonization with enteric microflora as well as marked changes in diet. Consequently, environmental factors could be a stimulus for CRIP upregulation observed in rats.

Changes in the microbial flora in the intestine are thought to play a role in the development of the ulcerative colitis associated with Interleukin-2 (IL-2) deficient mice (Sadlack et al., 1993) and the development of chronic enterocolitis in Interleukin-10 (IL-10) deficient mice (Berg et al., 1993). In both of these models, the mice develop an attenuated disease when they are kept in an SPF facility. Although it is commonly accepted that the enteric microbial flora is different for animals maintained in the SPF environment compared to the conventional environment, no particular intestinal pathogens were identified in the IL-10 deficient mice (conventional environment) which may have been responsible for the intestinal inflammation. This striking difference in symptoms in the IL-10 deficient

mice may have been attributable to some environmental factor(s) or other microorganism(s) that were not examined. The differential expression of the CRIP gene between conventional and SPF environments are also similar in direction to the responses seen in induction of chronic intestinal inflammation after null mutations (knock out) of the IL-2 and IL-10 genes (Sadlack et al., 1993 and Berg et al., 1993). This relationship suggests stimuli that influence IL-2 and IL-10 production may influence CRIP expression. Furthermore, CRIP expression correlates with anti-inflammatory and immunosuppressive activities, and thus may be a factor in these events.

In our studies, we also focused on phenotypic changes produced in the transgenic and non-transgenic mice, including body and organ weights, blood analysis, and the response to endotoxic challenge. Significant differences were observed in CRIP expression between animals raised in the different environments. These differences were more marked for non-transgenic mice. Interestingly, there was no effect of environment on the body or organ weights of either strain of these mice. If CRIP synthesis is altered by environment, this does not have a direct effect on growth during the time frame of our study, where body and organ weight was monitored for the first 8-12 weeks of life. If growth is affected by environment in the CRIP-Tg mice, a later time point may need to be examined. In IL-10 deficient mice, growth was normal up to 12 weeks. However, in this

regard, CRIP-Tg mice maintained as adult breeding stock have not shown any abnormality in breeding or growth.

Environment did appear to play a role in the alteration of certain blood paramenters. When CRIP-Tg mice were living in the SPF environment, there was a significant decrease in the percentage of lymphocytes. Whether the decrease in the number of lymphocytes is due primarily to a particular subset of lymphocytes (B-lymphocytes or T-lymphocytes) is not known. Previous examination of the thymocyte populations in CRIP-Tg and Non-Tg mice raised in the SPF environment demonstrated that the transgenic mice had a decrease in the number of single positive ($CD4^+/CD8^-$ or $CD4^-/CD8^+$) thymocytes (Davis et al., 1998). Whether the thymocyte population is altered in mice raised in the conventional environment was not examined in this study and will be a focus of future experiments. Differential cell counts did not reveal any alteration in total lymphocyte populations for mice in the conventional environment.

A decrease in the normal number of single positive or mature thymocytes in the CRIP-Tg mice may be related to the difference in response to LPS which was evident in the transgenic strain. This was the only group of mice to develop diarrhea in response to LPS challenge. This group was also able to produce more intestinal CRIP protein as compared to the Non-Tg mice raised in the conventional conditions.

LPS is known to cause a complex reaction of the immune system (Rietschel and Brade, 1992) and initially has strong stimulatory effects of macrophage and other immune cells. During the response, prostaglandins and leukotrienes are produced, and reactive molecules such as nitric oxide are released by LPS-stimulated macrophage. Cytokines such as IL-1, IL-6, and TNF-α are released to the surrounding tissue or serum, leading to the acitivation of B- and T-lymphocytes. Cytokine production in serum was monitored after LPS challenge in the two genotypes from the conventional environment. CRIP overexpression lead to an increase in the Th2 cytokines IL-6 and IL-10 and a decrease in the Th1 cytokine IFN-γ. There was no change in TNF-α. CRIP overexpression appears to alter cytokine patterns in CRIP-Tg mice with a shift to Th2 cytokine production. It is also interesting that TNF-α production is not changed with CRIP overexpression. The differences in the abilities of these mice to handle an LPS challenge is more strongly associated to shifts in IL-10, IL-6, and IFN-γ production. With IL-10 increasing so markedly (nearly 3 fold), the ability of this cytokine to suppress lymphocyte and macrophage function may explain in large part the increased sensitivity to LPS that the CRIP-Tg mice display.

The relationship of acute or chronic infection, nutritional status, and the occurrence of acute diarrhea remains a complicated and important question. Perhaps an understanding CRIP's role in the mechanisms that

produce LPS-induced diarrehea in the Tg mice, will produce an explanation for its broader role in immune function. Currently, there is a variety of data supporting zinc therapy for the treatment of acute diarrhea (Fuchs, 1998). Consequently, the zinc binding properties of intestinal CRIP (Hempe and Cousins, 1991) may be related to this benefit of supplemental zinc.

The spleen and thymus are organs important for the development of B- and T-lymphocytes. Alternatively, the intestine is the largest immune tissue in the body, facing large quantities of antigens on a daily basis. The intestine has adapted to changes in the environment over time such that this immune tissue performs two major functions: (1) immune exclusion and (2) oral tolerance (Brandtzaeg, 1998). The latter is a complex and poorly understood process which involves both local and systemic regulatory mechanisms to avoid hypersensitivity to dietary and microbial antigens. CRIP's overexpression appears to strongly influence the response of our mice to endotoxin. Therefore, if CRIP is involved in some immune pathway in the intestine, perhaps in an immunosuppressive manner similar to oral tolerance, this may explain the differential response to LPS between CRIP-Tg and Non-Tg mice observed in these experiments.

Like other LIM-only proteins, CRIP may be involved in control of cellular differentiation or proliferation. The LIM-only protein MLP is involved in muscle cell differentiation (Arber et al, 1994) and LMO1 (previously Rbtn1

or Ttg-1) is a transcription factor involved in cell proliferation. More recently the LIM-only protein LMO2 (previously Rbtn2 or Ttg-2) was found to be important for mouse hematopoietic development (Yamada et al., 1998) and is part of a DNA-binding complex which is necessary for this development (Wadman et al., 1997 and Neale et al., 1995). This may involve protein-protein interactions that include cytoplasmic factors and/or nuclear factors such as the nuclear LIM interactor protein (Jurata et al., 1996) or the Clim/Ldb/Nli coregulator family (Sugihara et al., 1998). The response of CRIP expression to the environment and LPS challenge, further strengthens a role for this LIM protein in down regulation of cellular host defense, possibly by controlling immune cell differentiaion. Further investigation of specific regulatory factors will be necessary in order to learn more about CRIP's specific function. In addition, since the CRIP gene appears to be related to immunosuppressive events, its zinc fingers may eventually be a target for drugs designed to stimulate the immune system (Rice et al., 1995 and Turpin et al., 1996).

CHAPTER 6
IMMUNE CHALLENGE

Influenza Virus Challenge

Influenza and pneumonia are the fourth leading cause of death in persons 65 and older in the United States. Because of the severity of this infection and the increases in the elderly population in this country, there is a great deal of research which focuses on understanding the infection process.

At present, research studies performed in influenza-challenged aged mice have pointed to a decline in cytotoxic T-lymphocyte activity (Bender et al., 1991), a decrease in Th1-associtated clearance mechanisms (Taylor et al., 1997), and a dysregulation of Th2 cytokine production associated with age. In young influenza virus-infected mice (8-10 weeks) the Th2 cytokine IL-4 caused a highly significant ($P < 0.001$) delay in virus clearance (Moran et al., 1996). A similar observation was made in T-cell clones from influenza-primed BALB/c mice (Graham et al., 1994). Researchers in this group concluded that Th2 T-cells may lead to immune mediated potentiation of injury during influenza infection. Evidence is mounting that shows a correlation between T-helper cell function changes (in addition to the more

commonly accepted changes in cytotoxic T-cell function) and delayed viral clearance in aged animals. The full significance of this work with mice in relationship to humans is not clear.

In CRIP-Tg mice, there were considerable differences in cytokine production (changes in both Th1 and Th2 cytokines) after LPS challenge as compared to Non-Tg control mice. Because LPS is considered to be an extracellular pathogen, we chose to challenge the mice with influenza virus, an intracellular pathogen. In this manner, we could evaluate the effect of CRIP overexpression on two different types of host immunity. If the CRIP-Tg mice responded to influenza virus-challenge in a different manner compared to challenged control mice, a possible explanation for this difference might be alterations in cytokine synthesis.

In this group of experiments, CRIP-Tg and Non-Tg control mice were infected with influenza A/Port Chalmers/1/73 (H3N2) virus and observed for up to 8 days. The mice were monitored for changes in body weight, serum zinc, and lung viral titers. In a separate group of experiments, splenocytes were isolated from non-challenged mice to observe response of the splenocytes to mitogen challenge. Mitogen proliferation and cytokine production were measured.

Materials and Methods

Mice Young (7-9 weeks) CRIP-Tg and Non-Tg mice from the SPF environment were used for this group of experiments. The mice were age and sex matched. The mice were infected intranasally with 20 μl virus (Influenza A/Port Chalmers/1/73 (H3N2)) or vehicle after pentobarbital (1.5 mg i.p.) anesthesia. The mice were observed over the next eight days. Body weight was monitored for each mouse over this period. Groups of mice were killed on days 2, 4, and 8 post infection. In a separate group of experiments, CRIP-Tg and Non-Tg mice from the conventional environment were killed and their splenocytes were isolated as described below. This group of mice was used for determining levels of IL-2 production in splenocytes after mitogen challenge.

Lung viral titers Viral titers were determined at the Geriatric Research, Education, and Clinical Center at the University of Florida under the direction of Dr. Bradley Bender according to established methods (Taylor et al., 1997). Briefly, lung tissues from the mice were collected on each of the days listed above. Viral titers were determined by inoculation of ground samples into Madin Darby canine kidney (MDCK) cells (2×10^4 cells/well) in 96-well plates. The titers were calculated using the method of Reed and Muench (1938).

Cytokine Assays Splenocyte IL-2, IL-10, IL-6, TNF-α, and IFN-γ production after mitogen (phytohemagglutinin, PHA) stimulation was quantified using commercial ELISA kits (Cytimmune, College Park, MD) or bioassay (Langkamp-Henken, et al., 1998).

Splenocyte Isolation and Mitogen Challenge Splenocytes were isolated by mincing mouse spleens and pressing the tissue fragments through a fine mesh nylon screen. Cells were washed with RPMI- (Mediatech, Cellgro, Herndon, VA) complete medium (RPMI-1640 supplemented with 50 U/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 25 mM Hepes Buffer, 100 μM 2-mercaptoethanol). Red blood cells were lysed by exposing the cells to 0.034 M saline for 1 min, followed by 0.274 M saline. After washing the cells for a total of 3 washes in RPMI-complete, the cells were suspended in RPMI-complete with 10% heat-inactivated fetal calf serum (FCS), counted and resuspended at 4×10^6 cells/ml in RPMI-complete with 10% FCS. An aliquot of cells (2×10^6 cells/ml) was incubated with 10 ug/ml of PHA in 48-well cell culture plates for 24 h at 37°C, 95% humidity, and 5% CO_2. The supernatants were removed and frozen at -80°C until the cytokines were quantified.

IL-2 Production Murine HT-2 lymphocytes (IL-2 dependent, 4×10^3 cells/well) were plated in triplicate in flat bottom 96-well microtiter plates. Supernatants of the splenocyte media from the above cultures or

recombinant IL-2 were added to each well. The plates were incubated for 19 h at 37°C, 95% humidity, and 5% CO_2. At this point, the cells were pulsed with [^{3}H]thymidine (6.7 Ci/mmol) for 5 h, then harvested with an automated cell harvester onto glass fiber filters. The samples were counted in a liquid scintillation counter and counts per minute (cpm) were considered to be proportional to HT-2 cell growth. Growth of the IL-2 dependent T-cells was proportional to the amount of IL-2 added to the standard curve wells.

Proliferation Assay Splenocytes were assayed as described above. An aliquot of cells (1 x 10^6 cells/ml) was incubated with 10 ug/ml of PHA in 96-well cell culture plates in a final volume of 200 μl for 72 h at 37°C, 95% humidity, and 5% CO_2. Cells were pulsed, harvested, and counted as described above. The data are presented as mean cpm [^{3}H]thymidine uptake of triplicate cultures.

Statistical Analysis Regression analysis (Excel) was used to determine IL-2 concentrations. For all other cytokines, regression analysis was performed using the microtiter plate reader software (Softmax, Molecular Devices, Sunnyvale, CA) to determine absolute amounts of cytokines in the unknowns. Tests for significance were performed using the InStat Biostatistics software package (GraphPad Software, San Diego, CA) or the

SAS System (SAS Institute Inc., Cary, NC). When necessary, data were subjected to $\log_{10}$ transformation to achieve homogeneity of variances.

Results

Influenza Virus-Challenge Blood was collected from the mice on days 2, 4, and 8 and serum was isolated (Figure 6-1). Serum zinc concentrations in both CRIP-Tg and Non-Tg mice average approximately 1.0 μg/ml. At day 2, the concentration was depressed in both genotypes to approximately 0.8-0.9 μg/ml. Serum zinc remained at this level for the Non-Tg mice through day 8. However, there was a subsequnet and significant decline ($P < 0.05$) in serum zinc in the CRIP-Tg mice by day 8.

Body weight was also monitored after influenza challenge over the same time period (Figure 6-2). All mice began losing weight during the first 24 h post-challenge. By day 3 the Non-Tg mice were starting to regain weight. The control mice continued to gain weight until day 5 when they were back to their initial body weight. The CRIP-Tg mice continued to lose weight through day 4. These mice did not regain weight and had significantly lower ($P < 0.05$) body weight than the control mice for the remainder of the study period. The ability of the mice to clear influenza virus from the lung was compromised in the CRIP-Tg genotype (Figure 6-3). The CRIP-Tg mice still had significantly more ($P< 0.05$) influenza virus in the

lung at day 4 compared to the Non-Tg controls. By day 8, all of the Non-Tg mice had cleared the virus from their lungs. In contrast, only one third of the CRIP-Tg mice cleared the virus and the others were not able to clear the virus (accounting for the large SEM for this data point). In summary, the influenza virus-challenged CRIP-Tg mice had lower serum zinc, lost more weight, and could not clear virus as well as compared to the Non-Tg controls.

Splenocyte cytokines After the splenocytes were challenged with the mitogen PHA, their ability to proliferate was measured (Figure 6-4). There is a direct relationship between ^{3}H-thymidine incorporation and splenocyte proliferation. Two separate groups of experiments were performed. There were highly significant decreases in splenocyte proliferation for both trial 1 and trial 2 ($P < 0.008$ and $P < 0.007$, respectively) for the CRIP-Tg splenocytes as compared to the Non-Tg splenocytes.

Cytokines were measured in the splenocyte culture supernatants 24 h after PHA challenge (Table 6-1). There was a significant decrease in IL-2 and IFN-γ ($P < 0.03$ and $P < 0.02$, respectively) in the CRIP-Tg mice. This was accompanied by a significant increase in IL-10 and IL-6 ($P < 0.04$ and $P < 0.005$, respectively) in the CRIP-Tg mice. There was no significant difference in TNF-α production between the two strains of mice.

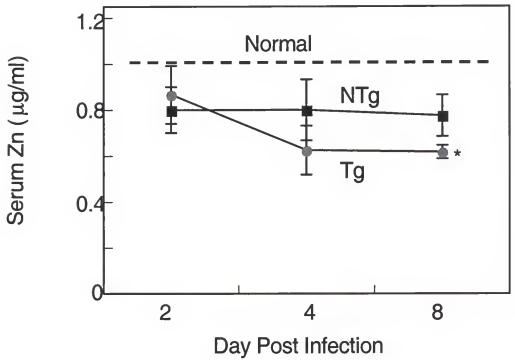


Figure 6-1. Comparison of serum zinc concentrations in transgenic and nontransgenic control mice after influenza challenge. Mice were challenged intranasally with influenza virus and groups were killed at 2, 4, and 8 days post infection. Serum zinc was measured using atomic absorption spectrophotometry. NTg, nontransgenic control mice;Tg, CRIP overexpressing transgenic mice. Values shown are means ± SEM, n=3 mice each value. *Statistically ($P < 0.05$) different from NTg control mice.

Table 6-1. Splenocyte cytokine production in transgenic and nontransgenic control mice after splenocytes were challenged with phytohemagglutinin (PHA).

Cytokine	IL-2 (U/ml)	IFN-γ (ng/ml)	IL-10 (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)
Genotype					
Non-Tg	0.69 ± 0.02	102 ± 31	7 ± 2	365 ± 58	411 ± 47
CRIP-Tg	0.56 ± 0.02	19 ± 7	17 ± 4	660 ± 56	401 ± 49
P value	0.03	0.005	0.003	0.03	N.S.

Values shown are mean ± SEM, n = 6 mice each value. Cytokines were measured in the splenocyte culture supernatants 24 h after PHA challenge. N.S., not significant

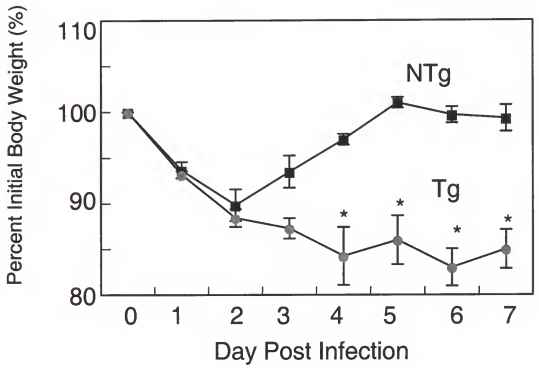


Figure 6-2. Comparison of body weight change in transgenic and nontransgenic control mice after influenza virus infection. Mice were challenged with influenza virus and body weight was monitored over the next eight days. NTg, nontransgenic control mice;Tg, CRIP overexpressing transgenic mice. Values shown are mean ± SEM, n=3-9 mice each value. *Statistically ($P < 0.05$) different from NTg control mice.

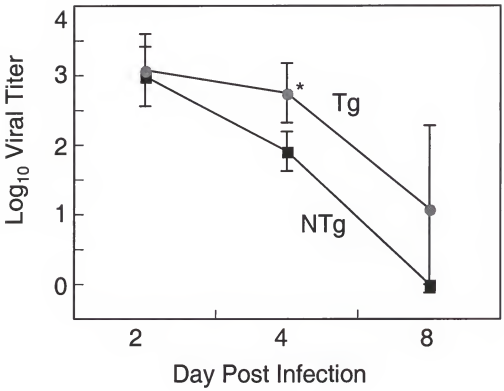


Figure 6-3. Comparison of lung viral titers in transgenic and nontransgenic control mice after influenza virus infection. Mice were challenged intranasally with influenza virus and groups of mice were killed on days 2, 4, and 8 postinfection. Lungs were harvested for lung viral load determinations as described in the text. NTg, nontransgenic control mice;Tg, CRIP overexpressing transgenic mice. Values shown are mean ± SEM, n = 3 mice each value. *Statistically ($P < 0.05$) different from NTg control mice.

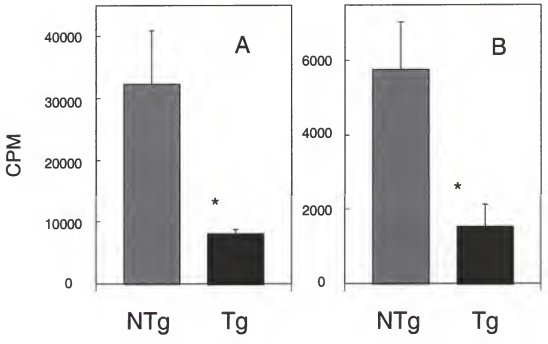


Figure 6-4. Comparison of splenocyte proliferation after mitogen challenge in transgenic and nontransgenic control mice. Splenocytes were isolated from each mouse and then incubated with the mitogen PHA. Cells were pulsed with ^{3}H-thymidine and then harvested onto glass filters. Non-challenged splenocytes were used as a blank control. NTg, nontransgenic control mice; Tg, CRIP overexpressing transgenic mice. Values shown are mean ± SEM, n=3 mice each value. A: experiment 1 proliferation($^{*}P < 0.008$), B: experiment 2 proliferation ($^{*}P < 0.008$).

Discussion

After evaluating splenocyte proliferation and cytokine production in mitogen-challenged splenocytes from both groups of mice, it was apparent that CRIP overexpression is influencing cytokine production. As with aging mice, there appears to be a decrease in the Th1 cytokines (IL-2 and IFN-γ) and an increase in the Th2 cytokines (IL-10 and IL-6) in response to these immune challenges.

As mentioned previously, aged mice have a decrease in Th-1 associated cytokines which may be related to a decreased ability to clear influenza virus from the lung (Taylor et al., 1997). Additionally, Th2 cytokines may cause delayed virus clearance in young influenza-infected mice. Aged C57BL/6Nia mouse splenocytes ($CD4^+$) produce ten times more IL-10 as compared to young mice after *in vitro* stimulation (Hobbs et al., 1994). This was accompanied by increased IL-10 mRNA expression in the aged mice. Interestingly, cytokine mRNA and protein expression do not seem to be correlated in all studies (Sarawar and Doherty, 1994).

In this study, we measured cytokine expression at one time point (24 h) after stimulation of splenocytes. We saw changes in IL-10, IL-2, IFN-γ, and IL-6 production, but no change in TNF-α production. In the case of TNF-α, it may be that another time point needs to be examined before a difference, if any, can be detected. Cytokine measurements appear to vary

based upon the type of cytokine, the tissue in which the cytokine is measured, and the mitogen/virus being used to stimulate the cells. One time point was chosen in relation to IL-2 production which typically peaks at 18-24 h post infection in the spleen. A time course study might also be helpful in understanding the relationship between the Th1 and Th2 cytokines in the CRIP-Tg and Non-Tg mice.

To balance the immune response, there is cross-regulation between the Th1/Th2 cells (Constant and Bottomly, 1997; Muraille and Leo, 1998). For example, IL-4 drives Th2 T-cell differentiation and IFN-γ and IL-12 drive Th1 T-cell differentiation. IL-4 and IFN-γ exert antagonistic effects on each other (Paludan, 1998). Thus from the data collected in this group of experiments two questions arise: 1) is CRIP overexpression affecting T-helper cell **function**?, i.e. upregulating Th2 cell function and downregulating Th1 cell function or 2) is CRIP overexpression affecting the **differentiation** pathways of these two cell types. Conversely, the effect of CRIP expression may be related to a combination of these two events. There are certain experiments which can be performed to answer these questions.

One of the first steps would be to evaluate the production of certain other cytokines mentioned above. Measuring IL-12 and IL-4 would help to answer whether the differentiation pathways are possibly being affected. In these mice, if T-helper cell differentiation is being influenced by CRIP

overexpression during an immune reponse, we would expect to see a decrease in IL-12 (downregulating Th1 differentiation) and an increase in IL-4 (upregulating Th2 differentiation).

The functional aspect of the T-helper cells could be addressed in part by performing an enzyme-linked spot-forming (ELISPOT) assay. Data collected from this type of experiment would address whether differences in cytokine levels are related to a difference in the number of cells or the amount of cytokine being produced in each cell. For example, aged influenza-infected mice have a decrease in the frequency of IL-2, IL-10, and IFN-γ producing cells (Taylor et al, 1997). Consequently, the functional capacity of the cells is normal, there are just fewer of these types of cells.

The final question that arises out of this group of work is whether CRIP overexpression is in some way related to the aging process. CRIP levels in the intestine rise to adult levels at the time of weaning in the rat. However, there is little if any information on CRIP expression in older animals. It would be interesting to evaluate CRIP levels in young and old mice. Even more interesting might be to look at CRIP protein levels in older humans, possibly by looking at concentrations in peripheral blood mononuclear cells.

CHAPTER 7
ROLE OF DIETARY ZINC AND METALLOTHIONEIN PRODUCTION ON CRIP EXPRESSION

Introduction

Because CRIP and other LIM proteins are zinc finger proteins, it is crucial to understand the effect of dietary zinc on their abundance. Early work demonstrated that CRIP mRNA expression in the intestine of rats and in transiently transfected IEC-6 cells is not zinc dependent (Levenson et al., 1994b). This was in contrast to metallothionein mRNA expression which was directly affected by zinc. However, CRIP protein levels were not measured in those experiments. Since we have recently developed the ELISA technique for CRIP protein quantification, we have the opportunity to ask questions such as whether the zinc content of the diet affects CRIP protein abundance in tissues. Furthermore, since we have a line of transgenic mice which overexpress the rat CRIP gene (Davis et al., 1998a), it was of interest to test whether the transgenics can overexpress CRIP protein when zinc is deficient in the diet.

Of equal interest is which cellular zinc pools help to regulate CRIP protein levels. Metallothionein represents one measurable intracellular pool

of zinc. Consequently we tested whether the zinc-binding protein metallothionein regulates CRIP protein levels. Specifically, we used MT-null (knockout) mice to examine the consequence of MT gene deletion on CRIP levels induced by lipopolysaccharide (LPS). Kosa et al. (1995) have provided convincing data that the two zinc fingers of CRIP have dissimilar zinc binding affinities, with the CCHC finger having the more facile zinc affinity. These observations open the possibility that one or both of the fingers of the LIM domain exchange their zinc with intracellular ligands in a coordinated or non-coordinated fashion. As mentioned previously (Chapter 1) recent studies have provided evidence that metallothionein is involved in the donation of zinc ions to apoproteins (Maret, 1998; Roesijadi et al., 1998)

Materials and Methods

Animals and Dietary Treatments Male and female CRIP-Tg and Non-Tg mice were age (all approximately 4-8 weeks old) and sex matched for each dietary group. All mice were housed and fed individually and handled in a similar manner as previously described for rats (Blanchard et al., 1996). All mice were fed an AIN-76A pelleted diet (Research Diets, New Brunswick, NJ; 5 mg Zn/kg diet) for the first week. At this point, the mice were randomized to one of three comparison groups. The first group was allowed free access to a purified diet which contained 30 mg Zn/kg diet (Zn

adequate group). A second group freely consumed a diet containing <1 mg Zn/kg diet (Zn deficient group). The third group consumed diet containing 30 mg Zn/kg diet and was pair-fed to a partner in the zinc deficient group. The mice were maintained for 21 days as described. All mice were given free access to glass-distilled, deionized water. The mice were weighed daily and food consumption was monitored. For methallothionein/CRIP expression experiments, metallothionein knockout (MT-KO) and controls (The Jackson Laboratory, Bar Harbor, ME) were injected i.p. with LPS (*E. coli* Serotype 0127:B8; Sigma, 2 mg/kg body weight) or saline.

Tissue Collection and Processing At the end of the comparison period, the animals were anesthetized with methoxyflurane and killed by cardiac puncture. Serum zinc concentrations were determined by atomic absorption spectrophotometry (AAS). Intestine, spleen, and thymus were taken for CRIP protein analysis and pancreas (diet study), intestine, spleen, thymus, and liver (MT study) were collected for MT protein.

Northern Analysis Northern analysis was performed as previously described (Hallquist et al., 1996 and Blanchard and Cousins, 1996) with 10-20 μg of total RNA from intestine, spleen, and thymus. Rat CRIP and ß-actin cDNA probes were prepared by RAD-Prime Labeling (Gibco BRL) with ^{32}P-dCTP. Densitometry was performed using Intelligent Quantifier 2.1 (BioImage, Ann Arbor, MI) with ß-actin signal as the internal standard.

Protein Assays Total metallothionein protein was determined for the specified tissues using the cadmium (^{109}Cd) binding method (Eaton and Toal, 1982). CRIP protein was determined using the CRIP sandwich ELISA as previously described.

Western Blotting Equal amounts of cytosolic protein (100 μg) from spleen of LPS-treated MT-KO and controls were separated on a 15% or 7% Tris-Tricine SDS-PAGE gel as described previously (Fernandes et al., 1997). The resolved proteins were then electrotransferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA). Blots were blocked with nonfat dry milk, incubated with the rabbit anti-rCRIP peptide IgG (as previously described) or rabbit anti-mSp1 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) followed by goat anti-rabbit IgG conjugated to Horseradish peroxidase (Sigma). Enhanced chemiluminescence detection was used for visualization.

Statistical analysis Regression analysis (Softmax software, Molecular Devices Corporation, Sunnyvale, CA) was used to determine CRIP concentrations. Tests for significance were performed using a 2x2 factorial ANOVA. The InStat Biostatistics software package (GraphPad Software, San Diego, CA) or the SAS System (SAS Institute Inc., Cary, NC) was used for determining level of significance. When necessary, data were subjected to $\log_{10}$ transformation to achieve homogeneity of variances

Results

Diet Study

Zinc Status Serum zinc was significantly reduced ($p<0.05$) for mice on the zinc-deficient diet (Figure 7-1). There was no significant difference in serum zinc for the zinc-adequate or pair-fed mice. There was also no significant effect of genotype on serum zinc for any of the treatment groups. Pancreatic MT was also significantly lower ($p < 0.01$) in the zinc-deficient group (Figure 7-2). Mice in the zinc-adequate and pair-fed groups did not have significantly different amounts of MT in the pancreas. There was a significant effect of genotype ($p < 0.01$) for the zinc-adequate group only. The zinc-adequate Non-Tg mice produced nearly twice the amount of MT in the pancreas as compared to the CRIP-Tg mice. Food intake and body weight were also monitored during the comparison period and there was no significant effect of diet or genotype on either parameter (Figure 7-3).

CRIP protein and mRNA There was a significant decrease ($p < 0.01$) in the amount of CRIP protein in the intestine of the zinc-deficient and pair-fed groups (Table 7-1). There was no significant difference in intestinal CRIP protein between the zinc-deficient and pair-fed animals. There was no significant effect of genotype on intestinal CRIP protein in the intestine. In the spleen, CRIP protein abundance was affected in a different manner by

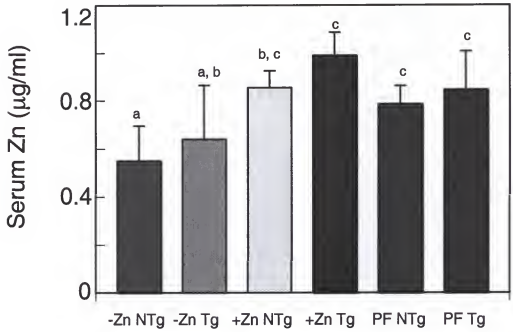


Figure 7-1. Comparison of serum zinc concentrations in CRIP-Tg and Non-Tg mice after zinc deficient diet (-Zn, < 1 mg Zn/kg diet) or zinc adequate diet (+Zn, 30 mg Zn/kg diet). Another group was pair-fed (PF, 30 mg Zn/kg diet) to the -Zn group. Serum zinc was measured using atomic absorption spectrophotometry. NTg, nontransgenic control mice; Tg, CRIP overexpressing transgenic mice. Values shown are mean ± SEM, n = 5 mice each value. Means with a different superscript are significantly ($P < 0.05$) different.

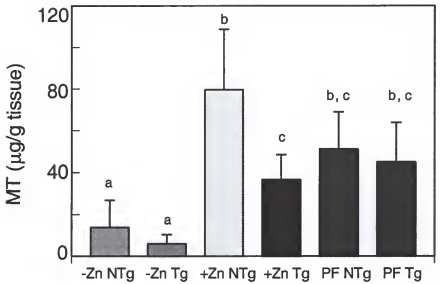


Figure 7-2. Comparison of pancreatic MTconcentrations in CRIP-Tg and Non-Tg mice after zinc deficient diet (-Zn, <1 mg Zn/kg diet) or zinc adequate diet (+Zn, 30 mg Zn/kg diet). Another group was pair-fed (PF, 30 mg Zn/kg diet) to the -Zn group. Pancreatic MT was measured using a cadmium binding assay as described in text. NTg, nontransgenic control mice; Tg, CRIP overexpressing transgenic mice. Values shown are mean ± SEM, n=5 mice each value. Means with a different superscript are significantly ($P < 0.01$) different.

diet (Table 7-1). There was a significant effect ($p < 0.002$) of diet in the CRIP-Tg mice only. The CRIP-Tg mice had less CRIP protein in the spleen when fed the zinc-deficient diet. The opposite effect was observed in the

Table 7-1: Effect of zinc deficient diet on CRIP protein in selected tissues.

Genotype/Diet		CRIP (µg/g total protein)	
	Intestine	Spleen	Thymus
-Zn NTg	49 ± 8^a	128 ± 22^a	41 ± 3^a
-Zn Tg	34 ± 6^a	41 ± 10^b	49 ± 9^a
genotype effect	NS	$p < 0.02$	NS
+Zn NTg	104 ± 19^b	86 ± 11^a	63 ± 10^a
+Zn Tg	96 ± 26^b	154 ± 35^c	54 ± 8^a
genotype effect	NS	$p < 0.04$	NS
PF NTg	49 ± 7^a	81 ± 25^a	50 ± 8^a
PF Tg	41 ± 4^a	$97 \pm 8^{a,b,c}$	32 ± 4^b
genotype effect	NS	NS	NS

Values shown are mean ±SEM, n=5 mice each value.
Diet effect: Means with a different superscript are significantly different intestine ($p < 0.01$); spleen ($p < 0.002$); thymus ($p < 0.01$).
Genotype effect: Means that are significantly different are indicated.
NS = no significant difference
NTg, nontransgenic control mice; Tg, CRIP overexpressing transgenic mice.
Zinc deficient diet (-Zn, <1 mg Zn/kg diet); zinc adequate diet (+Zn, 30 mg Zn/kg diet); pair-fed (PF, 30 mg Zn/kg diet) to the -Zn group.

spleens of the Non-Tg mice, but the difference was not significant. Additionally, there was a significant effect between genotypes in the zinc-deficient group ($p < 0.02$) and the zinc-adequate group ($p < 0.04$). There was no significant effect of genotype in the pair-fed mice. The least effect of genotype or diet on CRIP protein abundance was observed in the thymus. The only group of mice with significantly less ($p < 0.01$) CRIP protein in the thymus was the pair-fed CRIP-Tg group. Genotype had no effect on CRIP protein in the thymus.

The difference in CRIP mRNA levels clearly demonstrates the marked difference between the Non-Tg and Tg genotypes. This is most evident in spleen and thymus (Figure 7-4). Zinc intake was not a factor in expression which supports previous experiments which had focused on the intestine (Levenson et al., 1994b). There is a trend toward greater expression in the pair-fed mice, but these differences were not significantly different. Comparison of the CRIP mRNA levels and protein concentrations do not show any correlations. These data clearly demonstrate that CRIP expression is subject to considerable modification after mRNA processing. Such changes could include degradation of unneeded CRIP protein, intracellular redistribution (e.g., to the nucleus) or utilization, or possibly secretion.The LIM domain of CRIP is believed to function thru protein-protein

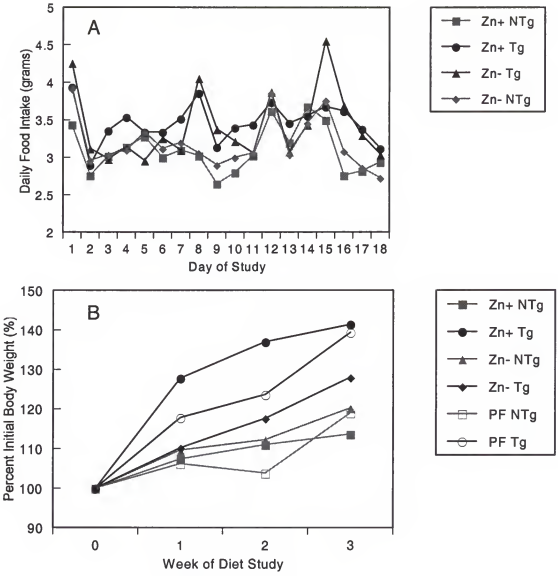
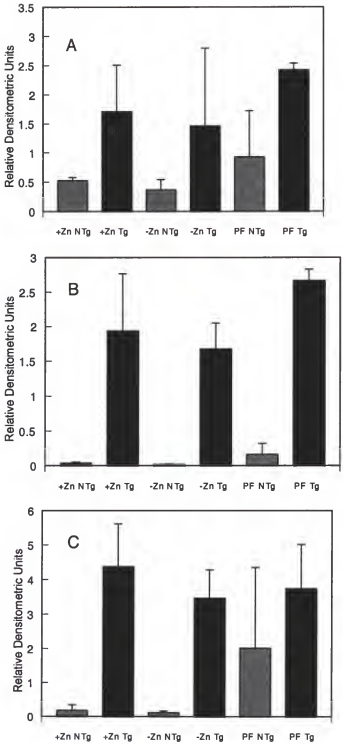


Figure 7-3. Food intake and body weight change in CRIP-Tg and Non-Tg mice during zinc deficient diet (-Zn, <1 mg Zn/kg diet) or zinc adequate diet (+Zn, 30 mg Zn/kg diet). Another group was pair-fed (PF, 30 mg Zn/kg diet) to the -Zn group. During the diet study, body weight and food intake were monitored daily. NTg, nontransgenic control mice; Tg, CRIP overexpressing transgenic mice. Values shown are means, n=5 mice each value. There was no significant effect of diet on these parameters. A: daily food intake; B: change in body weight.

Figure 7-4. Comparison of CRIP mRNA expression in CRIP-Tg and Non-Tg mice after zinc deficient diet (-Zn, <1 mg Zn/kg diet) or zinc adequate diet (+Zn, 30 mg Zn/kg diet). Another group was pair-fed (PF, 30 mg Zn/kg diet) to the -Zn group. CRIP mRNA was measured by northern analysis as described in text. NTg, nontransgenic control mice; Tg, CRIP overexpressing transgenic mice. Values shown are mean ± SEM, n=4-5 mice each value. Statistical analyses are described in text. A: intestine; B: spleen; C: thymus.


A
B
C
Relative Densitometric Units
+Zn NTg
+Zn Tg
-Zn NTg
-Zn Tg
PF NTg
PF Tg

interactions, thus each scenario above is currently possible based on literature on other LIM proteins. The mice used in these experiments were maintained in conventional housing which does, unto itself, produce some induction of CRIP in comparison to SPF conditions (Chapter 5). It is possible that an external factor, acute infection or trauma which alters the steady state, is necessary as a stimulus for closer correlations between dietary factors and genotype to be observed.

Metallothionein knockout study

Endotoxin response There was a very significant ($p < 0.0009$) reduction in serum zinc concentration following LPS challenge in the MT-KO and their controls (7-5). There was also a significant effect ($p < 0.03$) of genotype, with the MT-KO mice having higher serum zinc concentrations compared to the controls. Tissue metallothionein levels were significantly ($P < 0.05$) increased by LPS treatment in the control animals only (Table 7-2). There was a small amount of metallothionein in the MT-KO animals, as previously observed in our lab (Davis et al., 1998b). This is attibuted to non specific Cd-binding to other heat soluble cytosol constituents. However, there was not a significant increase in MT after the LPS-challenge.

CRIP protein Intestinal CRIP protein changed in response to the LPS challenge (Figure 7-6). There was a significant increase ($p < 0.003$) in the abundance of intestinal CRIP in the LPS-challenged control mice. In marked

contrast, intestinal CRIP protein did not increase in the LPS-challenged MT-KO mice. In order to evaluate the possibility of a more general effect of the MT null mutation, we looked at CRIP expression in other tissues of LPS-challenged mice (Figure 7-7). CRIP protein expression followed a similar pattern of expression as in the intestine of LPS-challenged mice. There was significantly less CRIP protein in the spleen ($p < 0.001$) and thymus ($p < 0.001$) of MT-KO mice relative to the controls or saline-treated mice. An attempt was made to measure CRIP expression in the liver of the mice, but CRIP protein was not detected in the samples. However, the abundance of CRIP in the liver is known to be very low (Davis et al., 1998b), especially relative to the thymus, spleen, and intestine. These results collectively show a dependence on MT expression when CRIP is induced by LPS. When there is no stimulus for an increase in CRIP expression, the lack of MT does not influence the cellular concentration of this zinc finger protein.

Western analysis Analysis of CRIP and Sp1 protein abundance in spleen demonstrated that CRIP is more abundant in LPS-treated controls as compared to LPS-treated MT-Knockouts (Figure 7-8). The transcription factor Sp1 was equally expressed in the spleen of both control and knockout mice after LPS treatment.

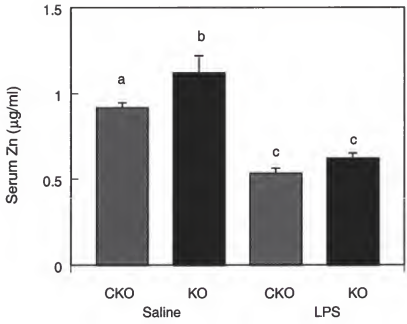


Figure 7-5. Change in serum zinc after LPS challenge or saline treatment in MT-knockout and control mice. Mice were challenged with LPS, killed 24 hours later, and serum was collected. Serum zinc was measured by atomic absorption spectrophotometry. KO, MT-knockout mice; CKO, control mice. Values shown are mean ± SEM, n=4-6 mice each value. Means with a different superscript are significantly ($P < 0.03$) different.

Table 7-2: Effect of LPS treatment on tissue metallothionein in MT-knockout and control mice.

Genotype	Control		MT-Knockouts	
		MT		
		(μg / g tissue)		
Tissue	Saline	LPS	Saline	LPS
Intestine	7 ± 1	10 ± 1*	6 ± 1	6 ± 1
Spleen	0.8 ± 0.1	4 ± 0.4*	0.5 ± 0.1	1.5 ± 0.6
Thymus	3.1	8.6	2.2	2.6
Liver	21 ± 4	100 ± 17*	9 ± 2	16 ± 2

Values shown are mean ± SEM, n=4 mice each value for intestine, spleen, and liver. For thymus, values shown represent pooled samples from n=4 mice. LPS, lipopolysaccharide, 0.2 mg/ kg body weight.
*Statistically ($P < 0.05$) different from saline-treated group.

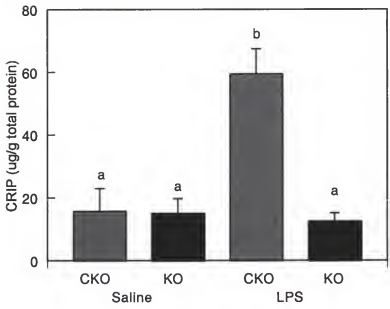


Figure 7-6. Intestinal CRIP protein concentrations after LPS challenge or saline treatment in MT-knockout and control mice. Mice were challenged with LPS, killed 24 hours later, and intestinal cytosol was prepared. Intestinal CRIP was measured by ELISA. KO, MT-knockout mice; CKO, control mice. Values shown are mean ± SEM, n = 4-6 mice each value. Means with a different superscript are significantly ($P < 0.003$) different.

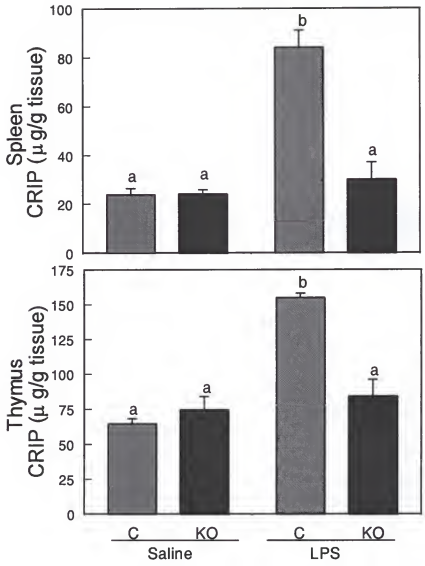


Figure 7-7. CRIP protein concentrations after LPS challenge or saline treatment in spleen and thymus of MT-knockout and control mice. Mice were challenged with LPS, killed 24 hours later, and tissue cytosol was prepared. Spleen or thymus CRIP was measured by ELISA. KO, MT-knockout mice; C, control mice. Values shown are mean ± SEM, n=4 mice each value. Means with a different superscript are significantly ($P < 0.001$) different.

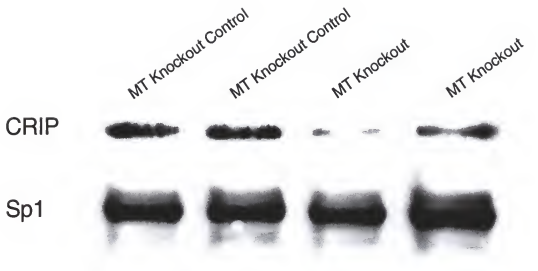


Figure 7-8. Measurement of CRIP and Sp1 proteins in spleen of MT-KO and controls by Western analysis. Cytosol fractions were separated by 15% (CRIP) and 7% (Sp1) Tris-Tricine SDS-PAGE and transferred to nitrocellulose membranes. Rabbit anti-rCRIP peptide IgG and rabbit anti-mSp1 IgG were used as detection antibodies.

Discussion

Data presented in this chapter show that tissue CRIP levels are not directly influenced by dietary zinc status but are dependent upon expression of metallothionein. Zinc deficiency was achieved after 3 weeks on a zinc deficient diet, as indicated by serum zinc and pancreatic metallothionein values. CRIP mRNA expression was not affected by dietary zinc restriction or energy restriction. However, CRIP protein expression was decreased in the intestine of zinc-deficient and pair-fed mice. CRIP protein expression in the spleen and thymus of the zinc-deficient and pair-fed mice appeared to be somewhat dysregulated.

With all tissues examined, there was no clear effect of dietary zinc, but rather a trend toward an effect of dietary modification. Although dietary zinc restriction can lead to decreased food consumption (O'Dell and Reeves, 1989), the zinc deficient mice and thus pair-fed mice did not consume less food in this study. A possible explanation for the dysregulation of CRIP protein levels in the pair-fed mice may be that the animals were affected more by the feeding method. These mice were fed a specified amount of food each day that was matched to the amount of food consumed by a paired zinc-deficient mouse. Perhaps the stress induced by the type of feeding in this group lead to dysregulation of CRIP protein levels.

Additionally, CRIP's possible involvement in immune function may help explain the changes in CRIP protein which were observed in the tissues. As demonstrated in Chapter 5, CRIP expression is influenced by environment. The group of experiments reported in this chapter were performed in the conventional environment which has been shown to stimulate CRIP expression in the intestine, spleen, and thymus. The fact that these mice were being stimulated by their environment during a state of zinc deficiency may help to rationalize the dysregulation in CRIP protein abundancy. Zinc deficiency and the possible energy restriction that it promotes has been associated with impaired function of T-cells and antigen-presenting cells in mice infected with gastrointestinal nematodes (Shi et al., 1998). Zinc deficiency and energy restriction were found to play distinct roles in regulating the host immune response. Clearly, the complexity of the factors which may be affecting CRIP in this particular study (zinc-restriction, energy-restriction, stress, host immunity) may be responsible for the dysregulation of CRIP protein observed here.

Because dietary zinc does not appear to be a strong regulator of CRIP expression, we also looked at the possibility that specific sources of zinc in the cell may affect CRIP abundancy. The MT-knockout mice utilized in this study presented as a good model to study the effect of metallothionein protein on CRIP expression during an immune challenge. As mentioned

above, LPS induces CRIP expression. However, it appears from this study that when metallothionein is not produced in the tissue (here intestine, spleen, or thymus), then CRIP protein is not increased by LPS.

It has been demonstrated that metallothionein and the apoprotein may be important in the regulation of enzymes involved in glycolysis and signal transduction (Maret et al., 1999). This process occurs as thionein (or apo-MT) removes zinc from an inhibitory zinc-specific enzymatic site. This removal of zinc results in an increase in enzyme activity. As mentioned previously, zinc can also be donated to apo-proteins from metallothionein. Zinc is bound rather tightly to MT with an association constant of 2×10^{11} M^{-1}. Depending on the relative association constants of other metalloproteins, MT may act as a zinc donor or zinc acceptor. Currently the association constant(s) for the zinc fingers of CRIP has not been determined and would be of great interest in relation to this study.

To evaluate the specificity of the CRIP/MT relationship observed herein, we chose to compare the ability of the MT-knockout mice to produce CRIP and another zinc-containing protein. The transcription factor Sp1 contains three zinc finger domains (Narayan et al., 1997). This is a sequence-specific transcription factor that recognizes GC boxes. It was initially identified as a HeLa cell derived factor that selectively activates in vitro transcription from the SV40 promoter. In this experiment, when MT-

knockout and control mice were challenged with LPS, the control mice were able to make more CRIP in the spleen as compared to the MT-knockout mice. The abundancy of Sp1 was not affected by lack of metallothionein.

Because the MT-knockout mice can still make CRIP, albeit the LPS-stimulated increase is not observed, there must be another pool of zinc which can donate the metal for CRIP synthesis. It may be possible to decrease this pool by placing the MT-knockout mice on a zinc deficient diet. After the zinc-restriction period, the mice would be challenged with LPS in order to stimulate CRIP expression. If the zinc pool is reduced by zinc deficiency and the mice cannot make metallothionein, CRIP production should be severly affected. This experiment may help to further understand the inter-relationship of CRIP and MT protein levels.

CHAPTER 8
SUMMARY, SPECULATIONS, AND CONCLUSIONS

Summary/Speculations

There has been a great deal of research surrounding cysteine-rich intestinal protein since discovery of CRIP cDNA in 1986 by Birkenmeier and Gordon. Because CRIP is a LIM-only protein, research has focused on the role of CRIP in development and immune function. Attempts have also been made to understand how CRIP interacts with other cellular proteins and factors. Although the specific function of CRIP is still unknown, there is increasing evidence supporting a role which is similar to that of other LIM-only proteins such as LMO2 or zyxin. The purposes of this study were to develop an immunochemical assay to measure CRIP and to generate data to help define CRIP's function.

The sandwich ELISA which was developed has proven to be a valuable assay for quantifying CRIP protein. The CRIP ELISA is efficient in terms of amount of sample needed and the time needed to perform the assay. It is unique in that it is the only ELISA used to measure a LIM protein. It is also one of the few quantitative assays for a zinc-finger protein. Finally, because of the similarity between the human and rodent

forms of the protein, the CRIP ELISA can be used to measure CRIP protein in tissues from both species. In this study, CRIP protein was quantified in tissues or cells from both humans and mice in order to learn more about CRIP's regulation.

The CRIP ELISA was initially utilized in measuring CRIP protein in human milk mononuclear cells (Chapter 3). From this group of experiments it was determined for the first time that CRIP was present in a population of human milk cells. Although the relationship between stage of lactation and CRIP protein levels was not very significant, there is a trend toward decreased expression with length of lactation. This longitudinal study involved a small number of subjects ($n=4$), and thus a greater sample size in future studies may be helpful. Another possibility for the study of CRIP in milk cells may be to utilize bovine milk samples. Because CRIP appears to be affected by immune challenges it would be interesting to evaluate CRIP expression in the mononuclear cells of milk from cow's with mastitis or in women with infections.

The other model which was utilized in this project was the mouse. The development of CRIP transgenic mice has provided an excellent model for further study of CRIP's expression and function. As part of my project, these mice were bred to the homozygous stage for use in studies involving immune function and dietary zinc. Because the mice overexpress the rat

CRIP gene, they are able to make significantly more CRIP mRNA and protein. An alternative to the overexpressing model would be a CRIP-knockout mouse. However, when other LIM proteins, such as LMO2 have been "knocked out," this results in a lethal mutation. With the more recent approach of cell-specific gene deletion, there may be a possibility of eventually creating cell- or tissue-specific CRIP knockouts.

A great deal of information about CRIP in an in vivo model was collected through the work with the transgenic mice. CRIP expression appears to be influenced by factors in the environment other than diet. In the study of environmental effects, the CRIP-Tg and Non-Tg mice were fed the same diet in different environments. The only differences which could account for the increase in CRIP expression in the conventional environment are environmental. We have not performed pathological examination of the mice in both environments to determine what pathogens or other factors may be affecting CRIP expression. A detailed examination for bacterial, fungal, or viral pathogens would be useful. Because CRIP expression in the intestine was most markedly affected by environment, it is possible that the pathogen is of enteric origin. This effect of environment must be a consideration when evaluating the data generated from various other studies in the mice.

Some of the most useful information about CRIP which we have generated from these studies is related to immune function. CRIP-Tg mice are more sensitive to both extracellular (LPS or PHA) and intracellular (influenza virus) pathogens. The CRIP-Tg mice lose more weight and have lower serum zinc levels compared to Non-Tg mice after challenge with these pathogens. The most striking pieces of data concern cytokine levels in the transgenic mice. When CRIP-Tg mice are challenged with LPS (in vivo) or their splenocytes are challenged with PHA (in vitro), there is an increase in the Th2 cytokines (IL-10, IL-6) and a decrease in the Th1 cytokines (IL-2, IFN-γ). The changes were most marked for IL-10 and IFN-γ. It is possible that CRIP is either affecting the differentiation of the cells which produce these cytokines or the function of the cytokine-producing cells. Experiments such as measuring the cytokines responsible for T-helper cell subset differentiation (IL-12 and IL-4) can be measured in the mice as discussed in Chapter 6. If differentiation of the T-cell subsets is being affected, it would be likely that IL-12 expression is decreased and IL-4 expression is increased in the CRIP-Tg mice.

An additional possibility may be that CRIP acts as a coregulator of cytokine synthesis. Whether CRIP would act as a corepressor or coactivator of the cytokines is not clear and would be interesting to evaluate. As pointed out earlier, the LIM-only protein LMO2 is a coregulator involved in

erythropoesis. It is interesting that the inability to initially meausure CRIP by ELISA occurred for specific tissues (spleen and thymus). This may point to a high abundance of tissue-specific protein-partners for CRIP in these tissues. A functional approach to measuring the effects of the shift in T-helper cell population is by evaluating delayed-type hypersensitivity (DTH) in the CRIP-Tg and Non-Tg mice. Because the Th1 cytokines are associated with the DTH response (Janeway and Travers, 1994), the CRIP transgenic mice should exhibit a decrease in DTH. A plethora of experiments could eventually be designed to measure ways in which to affect this shift in Th1/Th2 balance.

It would also be interesting to evaluate T-helper cell populations in relationship to CRIP abundancy in aged mice. The CRIP-Tg mice responded to influenza virus challenge in a very similar manner as aged mice respond. There has been more of a focus on CRIP expression in early development thus far. Perhaps evaluation of CRIP in aged mouse models would be useful in understanding CRIP's function and/or the decline in immune function which commonly occurs with age.

Collectively, the host immunity-related studies performed in this project have provided the framework for a model of CRIP expression in host immunity (Figure 8-1). In this model, it appears that when CRIP is overexpressed, there is a shift in helper T-cell balance. Overexpression of

CRIP stimulates a shift towards a Th2-like response. This type of response is normally stimulated by extracellular pathogens. We might speculate that when CRIP expression is depressed, there is a shift to a Th1 type response. This model helps support a role for CRIP in host immunity.

The final group of experiments in the study provide evidence that CRIP abundancy in cells may be related to metallothionein, particularly in cases where CRIP abundancy normally increases rapidly such as in an acute phase response. Metallothionein mice which cannot make MT-protein cannot produce extra CRIP protein after an LPS challenge as compared to control animals who have a marked increase in CRIP after the immune challenge. These mice have the ability to make a basal amount of CRIP protein, suggesting that other zinc pools normally contribute this metal for CRIP synthesis. These observations have been summarized in the model presented in Figure 8-2. As suggested in Chapter 7, the other zinc pools, the origins of which have yet to be defined, may be restricted by a zinc deficient diet, and in the MT-knockout mouse, CRIP synthesis after an immune challenge may be markedly reduced. These data are the first to demonstrate the MT-zinc finger relationship in an intact animal.

There does not appear to be a strong effect of dietary zinc restriction on CRIP. More likely, the dysregulation in CRIP in the intestine, spleen, and thymus may be related more to stress or feeding behavior. Because CRIP

appears to be strongly associated with immune function, and more specifically involved in some aspect of host immunity, it will be important to evaluate CRIP expression following an immune challenge in zinc-deficient mice.

Conclusions

Cysteine-rich intestinal protein is a zinc finger protein which is a member of the LIM-only family of proteins. Like other members of this family, it appears that CRIP may have a role in cellular proliferation or differentiation. Furthermore, this role may be a crucial aspect of host immunity and helper T-cell balance. CRIP is a zinc-requiring protein and the source of intracellular zinc may be dependent upon metallothionein during immune challgenges. Although the exact function of CRIP remains unknown, this study has provided further information to support a relationship of CRIP and host immunity. With the availability of the CRIP ELISA, further study of the protein will be facilitated.

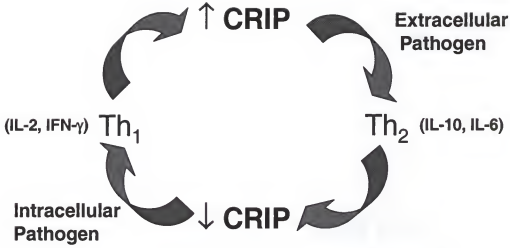


Figure 8-1. Potential role of CRIP in host Immunity and helper T-Cell response.

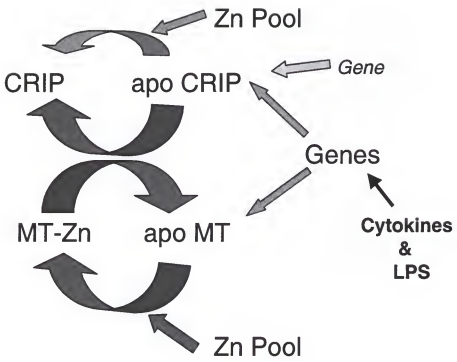


Figure 8-2. Potential relationship of intracellular CRIP and zinc binding via MT-Zn or other zinc pools.

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BIOGRAPHICAL SKETCH

Lorraine Marie Lanningham-Foster was born in Orlando, FL, on March 8, 1972, to Patricia Cato Lanningham and Bobby Lee Lanningham. She graduated from the North Carolina School of Science and Math in 1990 where she was a Glaxo Research Fellow. In 1990, she began her B.S. at the University of North Carolina at Greensboro in nutrition/dietetics with a minor in chemistry. She graduated with highest honors in 1994. Lorraine entered graduate school in 1994 and after working under George Loo, received her M.S. in nutrition science in 1995. Lorraine was married in 1994 to Randal C. Foster.

After moving to Florida in the summer of 1995, Lorraine began her Ph.D. studies at the University of Florida, Gainesville, FL,under the supervision of Robert J. Cousins. She is a current member of the Society for Experimental Biology and Medicine as well as Sigma Xi, National Research Honor Society. After completing her doctorate, Lorraine and Randy will be moving back to North Carolina where Lorraine will begin a Post-Doctoral Fellowship at Bowman Gray School of Medicine at Wake Forest University.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quantity, as a dissertation for the degree of Doctor of Philosophy.

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